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Significance of Intraluminal Thrombus in Abdominal Aortic Aneurysm

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To my family

A day without laughter is a day wasted - Charlie Chaplin

ABSTRACT

Most AAAs contain an intraluminal thrombus (ILT) and the volume of the ILT correlates with aneurysm size. The thrombus-covered wall is a common site for rupture after bleeding into the ILT.

The hypothesis for this thesis was generated when Kazi et al discovered differences in AAA wall between ILT covered and ILT free aortic wall. From computed tomography images of patients with abdominal aortic aneurysms containing an eccentric intraluminal thrombus, the aortic wall appeared to be partially in direct contact with the blood flow on one side, while the opposite aortic wall was covered by the ILT. In that study they concluded that the aorta portion situated under the ILT is thinner, with fragmented elastin fibers, apoptosis of smooth muscle cells and generally is more degraded compared to the AAA wall, which is not under the ILT. This characteristic difference was postulated to be due to mere presence of the ILT. In this thesis we investigated the role of the ILT in AAA by concentrating on the content of the ILT in terms of proteases and their activities and inhibitors.

We have collected ILT and AAA wall from 57 patients (14 women, 43 men) obtained during elective surgery and examined them by proteomic methods.

We have found that MMP1-2-9, and 13 and elastase and inhibitors, α -1 anti-trypsin, TIMP-1 and PAI-1 are present in the various layers of the ILT, but mostly active in the layer adjacent to blood flow. We also found significant amounts of MMP9 in complex with N-GAL in AAA tissue. Activity of MMP9 is preserved upon binding to N-GAL. Further, unpublished data show that membrane bound proteases are present and carried by microvesicles and are active in the ILT, particularly in the acellular layer of the ILT adjacent to the aortic wall.

In summary, ILT contains various active proteases. These proteases may contribute to degradation of the vessel wall and concomitantly lead to AAA rupture.

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- I Presence of N-GAL/MMP-9 complexes in human abdominal aortic aneurysms.**
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- II Protease activity in the multi-layered intra-luminal thrombus of abdominal aortic aneurysms**
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Atherosclerosis. 2011 Oct;218(2):294-9. Epub 2011 May 11.
- III Proteolytically Active ADAM10 and ADAM17 Carried on Membrane Microvesicles in Human Abdominal Aortic Aneurysms**
Maggie Folkesson, Chunjun Li, Siw Frebelius , Jesper Swedenborg, Kevin Jon Williams, Joy Roy, Per Eriksson, and Ming-Lin Liu
Manuscript

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ABBREVIATIONS

AAA	abdominal aortic aneurysm
Abl	abluminal
ADAM	<u>A</u> <u>D</u> isintegrin <u>A</u> nd <u>M</u> etalloprotease
CaCl ₂	calcium chloride
COPD	Chronic obstructive pulmonary disease
CT	computed tomography
EDP	elastase-derived peptides
ELISA	Enzyme-linked immunosorbent assay
EVAR	endovascular aneurysmal repair
FDP	fibrin/fibrinogen-degraded products
If	interface fluid
IHC	immunohistochemistry
ILT	intraluminal thrombus
JNK	c-JNK N-terminal kinase
LDL	low-density lipoprotein
Lum	Luminal
MASS	Multicenter Aneurysm Screening Study
Mid	Middle
MRI	magnetic resonance imaging
MMP1	matrix metalloproteinase 1
MMP2	matrix metalloproteinase 2
MMP9	matrix metalloproteinase 9
MMP13	matrix metalloproteinase 13
MV	microvesicle
nAChR	nicotinic Acetylcholine receptor
NE	neutrophil elastase
N-GAL	neutrophil gelatinase-associated lipocalin
NTh	non thrombus wall
NTVW	Non thrombus vessel wall
OR	open repair
PAI-1	plasminogen activator inhibitor type 1
P4H	prolyl-4 hydroxylase
SNP	single nucleotide polymorphisms
TAT	thrombin-antithrombin III complex

TACE	TNF α converting enzyme
TEM	Transmission electron microscopy
Th	thrombus wall
TIMP1	tissue inhibitors of metalloproteases 1
TIMP2	tissue inhibitors of metalloproteases 2
TIMP3	tissue inhibitors of metalloproteases 3
TIMP4	tissue inhibitors of metalloproteases 4
TNF α	tumor necrosis factor alpha
tPA	tissue plasminogen activator
uPA	urokinase plasminogen activator
TSE	tobacco smoke extract
TVW	thrombus vessel wall
US	ultrasonography
XDP	neutrophil elastase degraded product

PREFACE

Why do this?

A challenge:

A few years ago a folder with evidence collected around unresolved mysterious murders of elderly men and a few elderly women was given to me to investigate. Going through these files I noticed that all these people had a few things in common, they were aged and most of them smoked. Looking closer to the data collected through the years, one additional fact emerged. We observed that most of the victims had something called an Intra luminal thrombus (ILT) that seemed to be present at the crime scene. This structure had not received much attention but was considered a bystander and not involved in the murder conspiracy. Therefore I have taken upon myself to investigate this so-called “bystander” since it became more and more important to be able to solve this mystery by investigating the ILT.

It soon appeared that this was a big challenge to try to resolve the conspiracy between proteases and inhibitors in the ILT. I had some experience in working with murder cases but these were very different. This time the victims were mostly elderly men around 60-70 years of age. I accepted the challenge and hoped to be able to manage to add another puzzle piece to the vast mysterious deaths by abdominal aortic aneurysm.

Now after a few years working with these cases, we have managed to identify a few players that had taken part in this conspiracy. But the murderers are still out there....

INTRODUCTION

Definition

Merriam Webster dictionary: Greek *aneurysma* (ἀνεύρυσμα), from *aneurynein* to dilate, from *ana-* + *eurynein* to stretch, from *eurys* wide —Sanskrit *uru* broad, wide.

An aneurysm is an irreversible widening of a blood vessel and occurs in the aorta and other vessel, e.g., intracranial and popliteal arteries. The most common location is in the abdominal segment of the aorta (Figure 1), but aneurysms also occur in the ascending and descending thoracic aorta.

Human abdominal aortic aneurysm (AAA) and the intraluminal thrombus (ILT) that are generated in most AAAs are the foci of this thesis.

AAA is a widening of the abdominal aorta that can eventually rupture and cause immediate death.

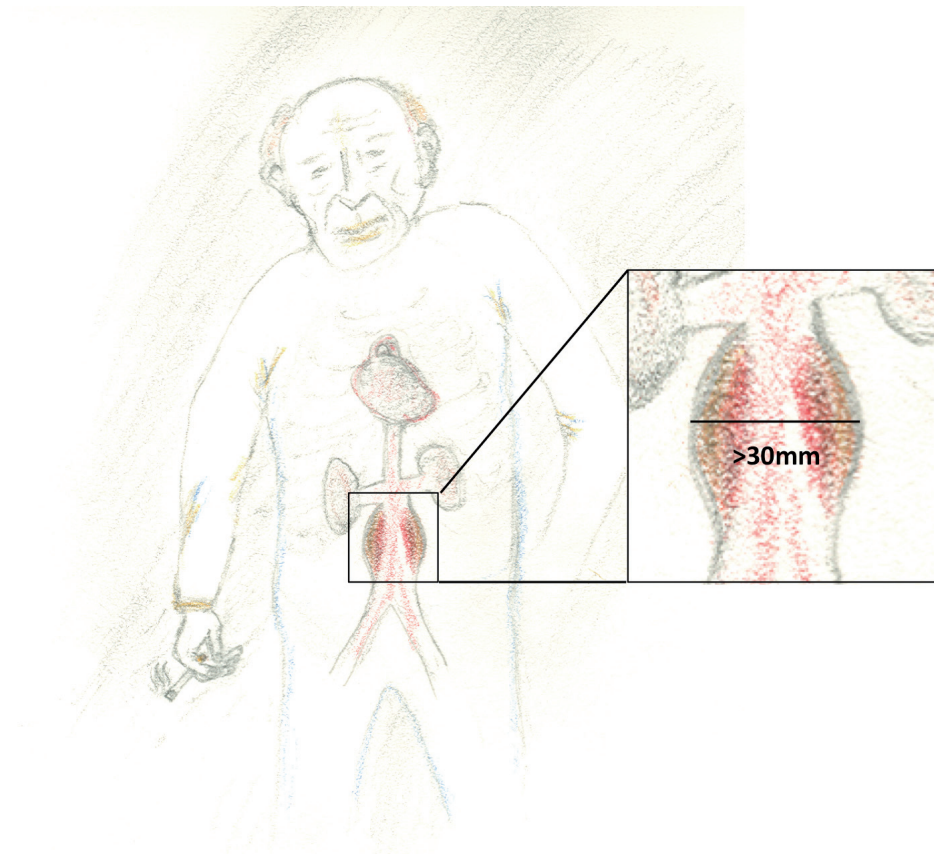


Figure 1: An image of an elderly male smoker with an abdominal aortic aneurysm >30 mm. Normal aortic diameter is 18.6-21.3 mm. (Allison, Kwan et al. 2008)(Yanasak, Allison et al. 2008)

There are several definitions of AAA in clinical use. One is when the diameter of the aorta is more than 50% larger than the diameter of adjacent normal aorta in that patient (Johnston, Rutherford et al. 1991) or 1.5 times the diameter of normal aorta in the general population (Upchurch and Schaub 2006). Another definition is when AAA diameter exceeds 30 mm. In clinical practice, the diagnosis of AAA may take in consideration that normal aortic diameter is dependent on age, sex, and body weight. For instance, the normal aortic diameter in women is smaller than in men (Sonesson, Lanne et al. 1994). These individual variations can be taken into account by defining the threshold for intervention when AAA diameter that reaches 1.5 times more than its normal size (Johnston, Rutherford et al. 1991). The most common threshold for intervention, however, is an absolute diameter of 55 mm or more (Lederle, Johnson et al. 2002), although this cut-off may place women at a greater risk for rupture. In Sweden, the 30-mm threshold is used as the definition of AAA, and 55 mm is the threshold for intervention.

The standard clinical methods for assessing abdominal aortic diameter are ultrasound, computed tomography (CT), and magnetic resonance imaging (MRI). Nevertheless, these methods can give somewhat different aortic diameters for the same individual. When the aortic diameter is close to 30 mm, even small discrepancies amongst these methods can mean the difference between a diagnosis of AAA, which will require ongoing surveillance for the rest of the patient's life, versus healthy aorta, which requires no surveillance. Therefore, it has been proposed that a more accurate and preferable definition of AAA should incorporate parameters for each individual, particularly the diameter of adjacent normal segments (Wanhainen 2008).

Abdominal aortic diameter 30mm is considered AAA.

Prevalence, heredity and genetics, and major risk factors

Prevalence

AAA is a common disease that causes 2% of deaths in Western countries (Nordon, Hinchliffe et al. 2011). Asians, American Hispanics, and blacks are less prone to develop AAA (Kent, Zwolak et al. 2010).

In the Multicenter Aneurysm Screening Study (MASS), 67,800 men in the UK between 65-74 years old were screened using ultrasound examination, during a 10-year period. In these individuals, a 4.9% prevalence of AAA was detected (Ashton, Buxton et al. 2002). Another screening study in Australia of 12,200 men between ages 65-83 showed a 7.2% prevalence (Norman, Jamrozik et al. 2004). A recent study by Svensjö et al. offered screening to every 65-year-old man in five Swedish counties. Over 22,000 accepted. The study concluded that the prevalence of AAA was the lowest of all previously reported studies (1.7% newly diagnosed and 2.2% total when including cases already under surveillance). By comparing their results with earlier studies, Svensjö et al. inferred that the prevalence of AAA has decreased in the past 30 years. The decrease could be due to the screening of 65 year-old men in contrast to previous studies where they included a wider range of elderly men e.g. 65-74 (Ashton, Buxton et al. 2002). They suspected this



was likely to be the substantial decrease in rates of smoking among this group of men (from 32% in 1980 down to 11% in 2007, Statistics Sweden). They also found an up to 4-fold association between AAA and smoking, independent of other risk factors (see *Smoking*, below). They propose screening of smokers in the future (Svensjo, Bjorck et al. 2011).

Heredity and genetics

The risk of AAA is doubled if the individual has an affected first-degree relative (Larsson, Granath et al. 2009), and approximately 15% of individuals with AAA have a positive family history (Darling, Brewster et al. 1989). Twin studies show a 10-fold higher risk to acquire AAA for monozygotic twins compared to dizygotic twins (Wahlgren, Larsson et al. 2010). Therefore it is a priority to screen first-degree relatives of AAA patients.

Because of the heritable component of AAA, roughly two decades of studies have attempted to associate genetic polymorphisms with this disease. Unfortunately, many of these studies were too small to be conclusive, and few of the associations could be replicated in independent cohorts (Hinterseher, Tromp et al. 2011). Large genome-wide association studies have discovered two single nucleotide polymorphisms (SNP) that consistently associate with AAA. The first is on chromosome 9q33, with an odds ratio of 1.21 ($P=4.6 \times 10^{-10}$). The SNP is within the gene encoding DAB2IP, which inhibits cell growth and survival. The study included 1292 patients with AAA size >30 mm and 30,530 controls who had not been screened. The same results were confirmed by a follow-up study of 3297 AAA patients and 7451 controls, all of European origin (Gretarsdottir, Baas et al. 2010). The second SNP associated with AAA is on 9p21, implicating the *ANRIL* gene, which regulates expression of two inhibitors of cyclin-dependent kinases (Hinterseher, Tromp et al. 2011). How variants in these two genes may lead to increased risk of AAA is not known (Hinterseher, Tromp et al. 2011).

Major risk factors

By far, smoking, male gender, and age are the major risk factors for AAA. Family history, discussed above, obesity, hypercholesterolemia, clinically apparent atherosclerosis in any vessel, and hypertension are comparatively weak risk factors, even though hypertension puts extra mechanical strain on the aortic wall (Brown and Powell 1999).

Smoking

Smoking is the most important environmental risk factor for AAA and the only risk factor that can be modified during disease progression. Chronic obstructive pulmonary disease (COPD), a condition caused primarily by smoking, is a predictor of AAA and may share mechanisms for matrix destruction (Murphy, Danna-Lopes et al. 1998).

Additional information about smoking:

There are about 2500 substances in tobacco, and tobacco smoke contains approximately 4000 chemicals, many of which are created during combustion. From 0.3% to 5% of the mass of tobacco consists of nicotine. Nicotine is an alkaloid, meaning a naturally occurring compound rich in basic nitrogens. Some alkaloids are naturally produced from amino acids. Serotonine, dopamine, and histamine are alkaloids.

Acetylcholines are natural signaling molecules that are ubiquitously found in all life forms. There are two main classes of acetylcholine receptors, one of which is called nicotinic (nAChR), because nicotine binds to this class of receptors and acts as a strong agonist. Particular classes of nAChRs exist in the brain, mediating addictive effects (Dome, Lazary et al. 2010), and on immune cells implicated in matrix damage in aorta and lung (Gahring and Rogers 2005).

Clearance of nicotine from the body in chronic smokers is slower than in occasional smokers. The most effective method to deliver nicotine into the reward centers of the brain is by tobacco smoke. Swallowed nicotine is not readily absorbed by stomach owing to the low pH of the gastric milieu. Nicotine and non-nicotine substances in inhaled smoke enter the alveoli of the lung and then nearly immediately cross into the bloodstream by passive diffusion. Several non-nicotine substances in tobacco smoke have been implicated in slowing the clearance of nicotine, thereby augmenting its effect. By contrast, intravenous administration of pure nicotine appears to be less addictive. Likewise, nicotine replacement products are only partially effective against addiction to tobacco smoking.

Each cigarette contains 10-14 mg nicotine, of which only 1-1.5 mg is inhaled and absorbed. The rest of the nicotine appears in side-stream smoke (not inhaled) or in between puffs (Dome, Lazary et al. 2010).

More than 80% of individuals with AAA are or were smokers. Current smokers experience a higher growth rate of AAA diameter than do former smokers (MacSweeney, Ellis et al. 1994; Brady, Thompson et al. 2004). Receptors that can bind nicotine are not only present on neurons but also on lymphocytes and macrophages (Gahring and Rogers 2005). Neutrophils exposed to nicotine increase their expression of elastase (Murphy, Danna-Lopes et al. 1998). Nicotine also activates matrix metalloproteinase (MMP2) expression by smooth muscle cells in the vessel wall, enhancing AAA formation in animals in vivo (Wang, Zhang et al. 2012). Studies in vitro indicate that tobacco smoke extracts cause death of human and animal smooth muscle cells, arterial and pulmonary endothelial cells, and human monocyte/macrophages (Raveendran, Wang et al. 2005; Hsu, Wu et al. 2009; Li, Yu et al. 2010). In a large cohort study, Kent et al. showed that the number of smoke-



years and total cigarettes smoked correlate with human AAA, and benefits from cessation of smoking were evident from their data (Kent, Zwolak et al. 2010).

Tobacco smoking is the strongest environmental risk factor for AAA, the only modifiable risk factor, and likely plays a direct causal role in the disease.

Gender and age

Other major risk factors are male gender, with a hazard ratio ≥ 1.97 for men relative to women, and age, with a hazard ratio ≥ 1.63 per half-decade (Rodin, Daviglus et al. 2003). The unadjusted gender ratio between men and women is about 5:1 (Lederle, Johnson et al. 2000).

In a population-based study in Norway, 6386 people between 25-84 years of age were recruited for ultrasound screening, which determined that 8.9% of the men and 2.2% of the women older than 48 years of age had AAA. The fact that no one under the age of 48 had AAA emphasizes the importance of age in this disease (Singh, Bonaa et al. 2001).

Obesity, high cholesterol and LDL levels, but also diabetes: Other studies show that excess weight correlated with increased risk for AAA (Cronin, Walker et al. 2012). In a recent study, Stackelberg et al. showed that body mass index has a weak relation to AAA, while abdominal adiposity with a threshold waist circumference of 100 cm for men and 88 cm for women was significantly correlated with the incidence of AAA (Stackelberg, Bjorck et al. 2012). Golledge and colleagues showed an association between serum resistin, a protein produced by adipose tissue, and AAA (Golledge, Clancy et al. 2007). Plasma concentrations of total cholesterol have been associated with AAA, with an odds ratio of 1.9 per mmol/L, and low-density lipoprotein (LDL) gives a somewhat stronger association, with an odds ratio of 2.3 per mmol/L (Wanhainen, Bergqvist et al. 2005). In another study, high plasma levels of LDL cholesterol were correlated with small aneurysms, suggesting a role for atherosclerosis in the initiation of the disease (Hobbs, Claridge et al. 2003). In addition, clinically apparent atherosclerosis in any vessel has been independently associated with AAA (Lederle, Johnson et al. 1997). Strikingly, diabetes has been shown to be protective against the development of AAA, possibly owing to increased arterial matrix synthesis, deposition of advanced glycation end products that inhibit matrix degradation, and use of angiotensin receptor blockers in these patients (Norman, Davis et al. 2007; Thompson, Cooper et al. 2010).

The risk of acquiring AAA is higher for patients suffering from other aneurysms. Popliteal aneurysm has been observed in 70% of patients with AAA (Wright, Matchett et al. 2004).

Smoking, advanced age, male gender, and family history are the most important risk factors for AAA.

Diagnosis and treatment

Enlargement of the diameter of AAA is the major prelude to rupture (Vardulaki, Prevost et al. 1998). Enlargement is associated with weakening of the aortic wall, and according

to Laplace's Law, the tension on the aortic wall increases with increasing diameter, even when the patient's blood pressure remains the same. Weakening of the wall combined with increasing mechanical tension drives further enlargement and eventually a catastrophic rupture of the largest artery in the body (Vorp and Vande Geest 2005).

Enlargement of AAA typically progresses for years without clear symptoms. Unfortunately, in many cases, the first symptoms occur during rupture, which is mostly lethal (see below). Thus, AAA has been called a silent killer. When symptoms occur before rupture, they can be nonspecific, such as lower back and side pain. The most diagnostic sign on physical exam is pulsation and bulging in the abdominal region of some patients.

Once the stress on the aneurysm wall exceeds its strength, rupture occurs. Rupture of AAA with extravasation of blood into the peritoneal cavity often leads to immediate death. However, in many cases the bleeding is confined by the limited capacity of the retroperitoneal space and is associated with improved odds of survival. The overall mortality for patients with an acute rupture is 80%, most of whom die before reaching a hospital. For those patients with an acute rupture who reach a hospital, the mortality rate for emergency surgical repair is 50%. By contrast, the mortality for elective repair of AAA is only 5% (Cota, Omer et al. 2005), despite the invasiveness of the procedures and the advanced age and typical comorbidities of these patients.

Until recently, the detection of AAA before rupture occurred by chance, either because the physician observed a pulsation in the abdominal region of the patient or the enlarged aorta was detected incidentally on abdominal imaging for another reason, such as evaluation of gallstones. Imaging modalities include computed tomography, ultrasound, and magnetic resonance imaging.

Several groups have attempted to identify biomarkers that are raised in the plasma of AAA patients compared to healthy controls (Urbonavicius, Urbonaviciene et al. 2008). D-dimer, a product of fibrinolysis, was found to be higher on average in AAA plasma compared to controls (Golledge, Muller et al. 2011). Other proteins that have also been shown to be up regulated in plasma of AAA patient are cytokines, such as IL6, IL-1 β and INF- γ , which the latter shows to be able to predict the expansion rate of AAA (Juvonen, Juvonen et al. 1997). Owing to limitations in sensitivity and specificity, particularly in predicting rupture, none of these proteins is used to diagnose or follow AAA patients today (Urbonavicius, Urbonaviciene et al. 2008).

Recently screening programs have been initiated, where every male of a certain age (usually 65 years or older) within a geographical region is offered examination by abdominal ultrasound. The screening permits detection of aneurysm that would otherwise go unnoticed before rupture. This type of screening saves lives and is cost effective (Lindholt, Juul et al. 2006). Some researchers have proposed that screening efforts focus on elderly and smokers. Screening of women is less common. In a contemporary study in Sweden, screening over 5000 women of 70 years of age, 19 were detected with AAA, of whom 18 were current or former smokers. This was only 0.4% of the total cohort and shows that prevalence of AAA in women is much lower than that in men (Svensjo, Bjorck et al. 2012) therefore screening of women is considered not to be cost effective (Ashton,



Buxton et al. 2002). It is known however that time-to-rupture is shorter for women than men (Wilson, Lee et al. 2003) and AAA related mortality rate for women is higher than men (Hultgren, Granath et al. 2007).

Patients with aneurysm diameter above 29 mm are offered surveillance by regularly scheduled ultrasounds. Rapid expansion of AAA is an important trigger for more frequent ultrasound examinations, because it is associated with increased risk of rupture (Limet, Sakalihassan et al. 1991). Patients in this group are offered elective surgical repair. In addition, ultrasound investigations are more frequent the bigger the diameter of the aneurysm. Regardless of the rate of expansion, when the aneurysm exceeds a threshold of 50-55mm in diameter, patients are offered surgery.

AAA patients rarely have symptoms until it is too late, and so screening programs have been established to detect AAA well before rupture.

Medical management

To prevent or slow the progression of AAA, the most important strategy is cessation of smoking, although the best evidence for this approach is epidemiologic, not from large-scale, prospective, randomized clinical trials (Golledge, Muller et al. 2006; Baxter, Terrin et al. 2008; Kent, Zwolak et al. 2010). In addition, anti-inflammatory agents (macrolides, statins), management of hypertension (propranolol, angiotensin-converting enzyme inhibitors, angiotensin receptor blockade), matrix metalloprotease inhibitors (doxycycline), and anti-oxidants have also been proposed, but evidence from large-scale clinical trials supporting these approaches is not available yet (Golledge, Muller et al. 2006) (Baxter, Terrin et al. 2008). As an example, tetracycline showed benefit in animals given elastase to induce a model of AAA (Pyo, Lee et al. 2000). Unfortunately, clinical trials in human AAA have been small and contradictory. Safety and benefit from long exposure time to doxycycline should be evaluated in larger cohorts (Dodd and Spence 2011). Regarding anti-inflammatory approaches, deletion of neutrophils (Eliason, Hannawa et al. 2005) or mast cells (Sun, Sukhova et al. 2007) showed benefit in murine models of AAA, and trials of anti mast cell drugs in human AAA patients have been proposed (Shi and Lindholt 2012). More research is needed to identify approaches to inhibit the progression of human AAA.

There are no conclusively proven medical approaches, only watchful surveillance and then surgery to help AAA patients.

Surgical treatment

Patients with AAA diameter exceeding 55 mm are offered elective surgery, which can be performed by open repair (OR) or endovascular aneurysmal repair (EVAR). Open repair involves exposure of the diseased aortic segment and surgical placement of a synthetic graft. Endovascular repair involves the introduction of a catheter via the femoral artery,

followed by placement of an endograft under X-ray guidance within the lumen of the diseased aortic segment. Several randomized clinical trials with large cohorts have monitored the outcomes from these two different methods and shown an initial survival advantage after EVAR, but similar long-term survivals after OR and EVAR (Lederle, Freischlag et al. 2009; De Bruin, Baas et al. 2010; Greenhalgh, Brown et al. 2010; Becquemin, Pillet et al. 2011).

The choice of method may depend on the anatomy of the aneurysm, the condition of the patient regarding cardiac and lung functions, and the policy of the care unit where the patient is treated. Endovascular repair may be preferred for patients unfit for open surgery, but requires regular, lifelong surveillance after the operation for possible leaks from the graft (called endoleaks). For AAA patients who are unfit for open surgery owing to comorbidities, the choice between endovascular repair and conservative non-operative management was addressed by randomized EVAR 2 trial, but not conclusively resolved (Rutherford 2006).

*Elective AAA repair is recommended for patients with
AAA diameter exceeding 50-55 mm who are fit for surgery.*

Pathophysiology

Anatomy of the normal aorta and regional features that predispose to aneurysm

The walls of every artery, including the aorta, consist of 3 layers: the tunica intima, tunica media, and tunica adventitia (usually called the intima, media, and adventitia). The intima is the innermost layer, right next to the flowing blood. This layer is composed of a single layer of endothelial cells anchored by connective tissue to the layer immediately below, which is the media. The media consists of smooth muscle cells and elastin fibers, collagens, and other connective tissue molecules. The third and outermost layer, the adventitia, consists of fibroblasts, various hematopoietic cells, neurons, small capillaries, and connective tissue. The cells of the aortic wall are provided with nutrition and oxygen via small capillaries called vasa vasorum. Aortic vasa vasora, however, are present in the thoracic region, but not below the renal arteries. Instead, the cells in the wall of the abdominal section of the aorta are supplied by diffusion from luminal blood.

Figure 2 shows a cross section of a normal abdominal aorta.



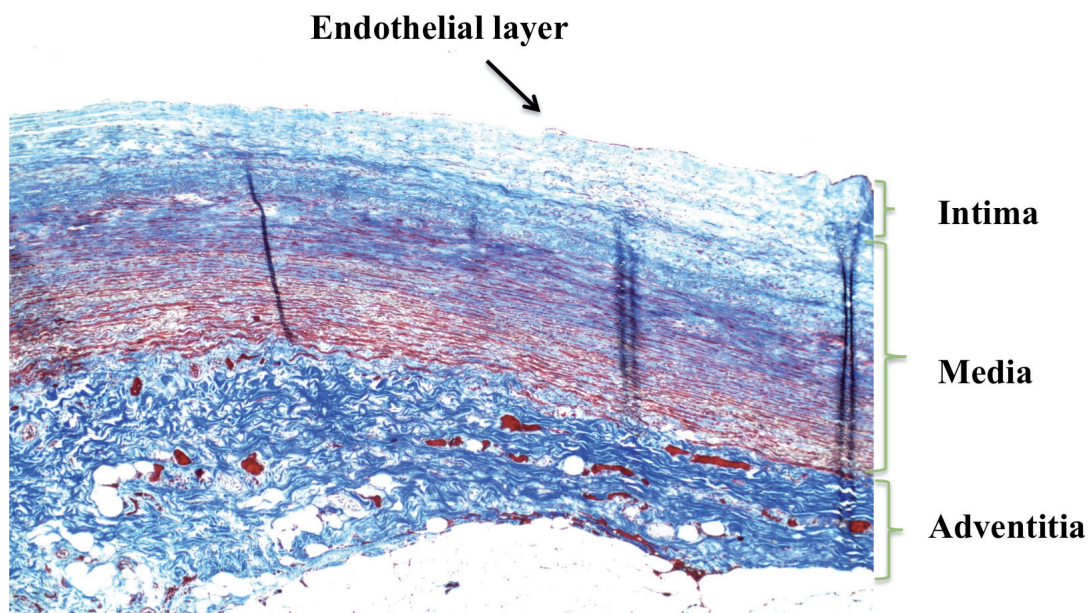


Figure 2: A section of normal aorta stained with Masson's Trichrome technique, in which smooth muscle cells appear red, collagen stains light blue, and the cell nucleolus appears dark blue. 10 times magnification.

It has been suggested that different segments of the aorta arise from different embryonic origins. In a study of murine embryos, vascular smooth muscle cells in various sections of the vasculature had different embryonic origins. In particular, the abdominal aorta diverges from the thoracic aorta. Smooth muscle cells in abdominal region of the aorta originate from mesoangioblasts, while smooth muscle cells from the abdominal aorta and femoral arteries originate from somites and various stem cells respectively (Jiang, Rowitch et al. 2000) (Majesky 2007). Whether the different origins of vascular smooth muscle cells affect the characteristics of these cells and the predisposition to aortic diseases are not known. Nevertheless, some authors have speculated that the embryonic origins of the abdominal aorta may partly explain why it is far more likely to become aneurysmal than its neighboring sections.

In addition, the amount of elastin is not uniform along the length of aorta. The further from the heart, the fewer the elastin fibers there are in the aorta (Ahmed 1968). This observation was first made in 1928, when Alfred Benninghoff investigated the amount of elastin along the length of the human aorta and discovered a decrease in the amounts of elastin and collagen from proximal to distal aorta. Benninghoff estimated 60-80 layers of elastin in the ascending aorta, but only 20 layers in the infrarenal aorta (Wolinsky and Glagov 1969).

In a later study, the infrarenal aorta was reported to have 58% fewer elastin layers, compared to the suprarenal aorta, and the infrarenal aorta exhibited the lowest proportion of elastin to collagen (Halloran and Baxter 1995). These observations provide an additional explanation why most aortic aneurysms are seen in the infrarenal region.

Further evidence for structural weakness in the abdominal aorta comes from a study, in which the researchers used ultrasound to compute wall stress in the abdominal aorta

and common carotid arteries of 111 healthy individuals between ages 25-70 years. Measurement of luminal diameter and intima-media thickness of these vessels showed that age was associated with increasing diameter of the all of these arteries, but was compensated by additional thickness of the wall of the common carotid arteries, and so wall stress in these vessels remained constant with age. Some wall thickening was seen in abdominal aortae of older men, but was insufficient to fully compensate for the stress from increased vessel diameter. Thus, wall stress in the abdominal aorta increased with age in men and may contribute to the development of AAA in elderly men (Astrand, Ryden-Ahlgren et al. 2005).

*The abdominal aorta has several features
that may predispose it to aneurysm formation.*

Cells in the normal and aneurysmal aorta

The normal aortic wall contains endothelial cells, smooth muscle cells, and fibroblasts. Histological studies show that segments of AAA are not lined with endothelial cells (e.g., see Figure 3). Aneurysmal vessels contain smooth muscle cells, but in decreased amounts (Kazi, Thyberg et al. 2003), and mRNA expression of alpha actin is lower in AAA vessels compared to healthy vessels (Mayranpaa, Trosien et al. 2009).

Importantly, AAA segments contain platelets and inflammatory cells, particularly macrophages, neutrophils, and mast cells. These cells are rarely found in normal aorta. Platelets together with inflammatory cells produce proteases that may further damage the vessel (Sakalihasan, Limet et al. 2005; Wohnner 2008).

Aneurysmal aorta contains inflammatory cells but is poor in smooth muscle cells.

A comparison between Figure 2 and 3 shows clearly that the aneurysmal wall is distorted, and the number of smooth muscle cells (red) is decreased substantially, while collagen (blue) is initially overproduced.



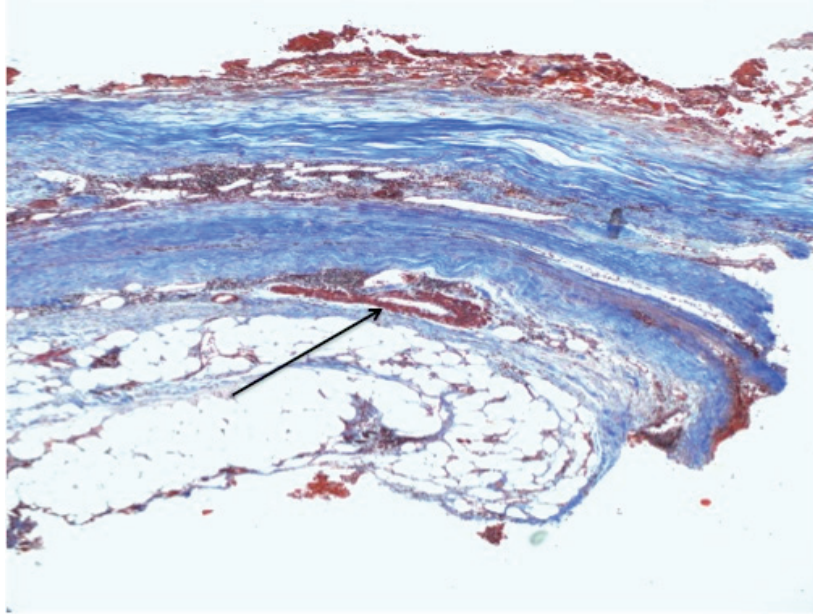


Figure 3: A section of aneurysmal abdominal aorta stained with Masson's Trichrome technique. Compared to the normal segment in Figure 2, smooth muscle (red) is decreased, and collagen (light blue) is increased. 10-times magnification. The arrow indicates neovascularization. The red region on the outer right surface is the remnant of an intraluminal thrombus, the majority of which had been removed during open repair.

Matrix of the aorta

There are several macromolecules comprising the extracellular matrix of the aorta. Among them are laminin, various types of proteoglycans, fibronectin, collagen, and elastin. The relevant macromolecules to this thesis are elastin and collagen types I and III. These molecules provide elasticity and strength to the aortic wall, enabling it to store energy by distending during systole, and then recoil back during diastole, thereby maintaining systemic blood pressure while the left ventricle is relaxing and the aortic valve is closed. While elastin provides physiological strength in the form of elasticity to the vessel wall, collagen is required for mechanical strength at higher pressure and provides rigidity to the vessel. It has been shown that canine models of AAA induced by treatment of the aorta with elastase show dilatation without rupture, whereas treatment with collagenases resulted in less dilatation but was followed by rupture (Dobrin, Baker et al. 1984).

Elastin provides elasticity while collagen provides strength to the aorta.

Elastin

Elastin, a macromolecule in the medial layer of the aorta, enables the vessel to absorb the pulsatile energy from the heart, to increase aortic diameter at systole and regain its initial shape during diastole. Elastin is produced at early stages of infancy and must last throughout lifetime of an individual. Production of elastin in the aorta later in life has not

been detected. Elastin has a high threshold for environmental assaults i.e. temperature and chemicals and has a half-life of 40 years (Rucker and Tinker 1977).

It has been suggested that the first event in the onset of AAA is loss of elastin (Thompson 1996; Jacob 2003). Histological studies show that AAA walls contain markedly reduced amounts of elastin, which is a result of the activity of elastases. The presence of neutrophil elastase (NE) originating from polymorphonuclear cells in AAA tissue has been shown to cause degradation of elastin (Fontaine, Touat et al. 2004). In addition, matrix metalloproteinases (MMPs) and cathepsins (Liu, Sukhova et al. 2005) also have the ability to degrade elastin (Herron, Unemori et al. 1991; Vine and Powell 1991; Thompson 1996). It has been suggested that all the above-mentioned proteases have a role in loss of elasticity of the aortic wall and the increase of AAA diameter (Pearce and Shively 2006; Allaire, Schneider et al. 2009).

Elastase-derived peptides (EDPs), which are produced by degradation of elastin, show an increased abundance in AAA compared to normal aorta (Petersen, Wagberg et al. 2002; Urbonavicius, Urbonaviciene et al. 2008). Moreover, EDPs have been shown to recruit inflammatory cells, which in turn produce more proteases that continue the degradation of elastin, leading to a vicious cycle (Satta, Laurila et al. 1998; Hance, Tataria et al. 2002). The imbalance between protease and inhibitor exacerbates the progression of AAA (Cohen, Mandell et al. 1988).

Two experimental mouse models of AAA rely on artificial damage to aortic elastin. In the elastase model, the infra-renal abdominal aorta of mice is briefly clamped off, porcine elastase is injected into the lumen, and after a brief incubation, the elastase is rinsed out and normal blood flow restored. This manipulation induces aneurysm formation and lasting within 2 weeks of the procedure (Thompson, Curci et al. 2006). In the calcium chloride (CaCl_2) model, the aorta is exposed to a CaCl_2 solution placed briefly on the adventitial surface. The CaCl_2 provokes an inflammatory reaction that disrupts the elastic network, leading to degradation of elastin (Chiou, Chiu et al. 2001). The aorta becomes aneurismal, but in the mouse, it is reversible, and the aneurysm regresses to its original size after 6 weeks.

AAA starts with degradation of elastin, and no new elastin is produced after infancy.

Collagen

Another major component of the connective tissue that gives the aorta its strength and rigidity is collagen. There are several types of collagen, but collagen types I and III are the predominant types in blood vessels. Degradation of collagens is a result of the activity of collagenases, such as MMP1, MMP8, and MMP13, all of which have been found in AAA tissue (Panek, Gacko et al. 2004). Collagenases are capable of initiating the degradation of intact, triple-helical collagen. Once collagen degradation has begun, a second set of enzymes, called gelatinases, are able to digest partially degraded, denatured collagen. Gelatinases include MMP2 and MMP9 (Elmore, Keister et al. 1998). Degradation of elastin and collagen triggers the production of collagen in the AAA wall (Carmo, Colombo et al. 2002; Abdul-Hussien, Soekhoe et al. 2007), and so there is an initial increase in



aortic collagen content (see Figure 3). As AAA progresses, however, the degradation of collagen overwhelms its production, leading to rupture (Lindeman, Ashcroft et al. 2010).

During the development of AAA, the structure of the vessel wall is disrupted and the wall weakens (Figure 3). At later stages, production of extracellular matrix is decreased owing to apoptosis of smooth muscle cells, while at the same time, continued degradation of elastin and collagen renders the AAA wall thin (Kazi, Thyberg et al. 2003). Rupture occurs when the components of the disrupted aortic wall fail to withstand the mechanical stress from pulsatile blood flow.

Collagens give the aortic wall its strength.

Proteases and their inhibitors

The proteases investigated in this thesis are introduced in Table 1, below, along with their inhibitors and known substrates.

Table 1: Proteases studied in this thesis, their inhibitors, and substrates (adapted from (Brew and Nagase 2010; Shiomi, Lemaitre et al. 2010)).

Protease	inhibitor	substrate
Elastase	α -1 antitrypsin	Elastin, Fibrin
MMP1	TIMP1-4	Collagens I, II, III, VII, X, entacin, aggrecan, perlecan, link protein, tenascin
MMP2	TIMP1-4	Gelatin, collagens IV, V, VII, X, XI, Fibronectin, Laminin, Elastin, aggrecan, link protein
MMP9	TIMP1-4	Gelatin, collagens III, IV, V, elastin, aggrecan, vitronectin link protein, N-telopeptide of collagen I
MMP13	TIMP1-4	Collagen I, II, III, IV, IX, X, XIV, Fibronectin, Laminin, Perlecan, tenascin, osteonectin
ADAM10	TIMP1-3	ProTNF- α , delta, collagen IV, gelatin Myelin basic protein, L1, APP, ProHB-EGF, CD44, Notch, Delta-like1, Jagged, N- and E-Cadherin, Ephrin A2, IL6R, Ephrin A5 Fas-L
ADAM17	TIMP1-3	ProTNF- α , ProTGF- α , TNF-p75 receptor, TRANCE, ErbB4, proHB-EGF, proepiregulin, proamphiregulin, IL6R, L-selectin, APP, CD44

Neutrophil elastase: its role in coagulation/fibrinolysis and its inhibition by α -1-antitrypsin

Neutrophil elastase (NE) is a serine protease produced by polymorphonuclear neutrophilic leukocytes (neutrophils) and stored in their azurophilic granules. This protein is released during inflammation and has the capacity to degrade extracellular matrix, especially elastin. It also has anti bacterial activity. Elastin derived peptides (EDP) in AAA attract neutrophils to the AAA site (Cohen, Keegan et al. 1991).

Coagulation factors, such as factor XIII, are degraded by elastase, while factors VII, VIII, IX, and XII are inactivated by elastase (Schmidt, Egbring et al. 1975; Nilsson, Holmberg et al. 1980). Normally, tissues are protected by inhibitors of this protease. The major

inhibitor for NE is α -1-antitrypsin, which is a serine protease inhibitor generated by the liver and released into the blood in high amounts. The other inhibitor of elastase is called elafin, which is produced by epithelial cells in lung and skin. Inflammatory processes and involvement of matrix metalloproteinases can block the activity of α -1-antitrypsin, despite its saturating concentrations in the blood (Sires, Murphy et al. 1994).

Cigarette smoke (Weitz, Crowley et al. 1987) and its major component, nicotine (Murphy, Danna-Lopes et al. 1998), increase NE activity and at the same time impair the ability of α -1-antitrypsin to inhibit elastin. These two effects lead to more activity of NE (Cohen and James 1982). Impairment in the ability of α -1-antitrypsin to inhibit NE is mediated by oxidation of one amino acid in the active site of α -1-antitrypsin, which makes its attachment to NE less effective (Li, Alam et al. 2009).

NE is capable of degrading fibrin in ILT (Kolev, Komorowicz et al. 1996). Fibrin, however, is degraded mostly by plasmin/mini-plasminogen, and the degradation products are different arrangements of 2 core fibrin fragments, fragment D and E. DD, DDE and E, with the major product being, DD also called D-Dimer. Plasma levels of D-dimer have been showed to correlate with growth of AAA (Golledge, Muller et al. 2011). NE degrades fibrin into a peptide, called the NE degraded product (XDP), that differs from D-dimer by a few amino acids and can be separately assayed (Kohno, Inuzuka et al. 2000).

NE is capable of cleaving plasminogen to “des-Kringles₁₋₄” also called mini-plasminogen. Mini-plasminogen activated to mini-plasmin in a faster rate than the activation of plasminogen to plasmin. Mini-plasminogen is resistant to inhibition compared to plasminogen and also more effective at degrading fibrin. Therefore, NE can degrade fibrin directly or indirectly by activating mini-plasminogen (Gombas, Kolev et al. 2004). Consequently neutrophils leucocytes associated with thrombi contribute to fibrinolysis (Kolev, Komorowicz et al. 1996).

Several fibrinolysis and coagulation factors, such as thrombin-antithrombin III complex (TAT) and fibrin/fibrinogen-degraded products (FDP) have shown to correlate with size of AAA (Yamazumi, Ojio et al. 1998). Another component of fibrinolysis investigated in AAA is plasminogen activator inhibitor type 1 (PAI-1), which is an inhibitor of fibrinolysis. PAI-1 acts by inhibiting tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA), which are activators of plasminogen (Louwrens, Kwaan et al. 1995). It has been shown that NE is capable of inactivating PAI-1, which is yet another mechanism to enhance fibrinolysis (Wu, Urano et al. 1995). This degradation will facilitate bleeding into the ILT and increases the risk of rupture that we discuss later.

Neutrophil elastase degrades elastin and fibrin and is active in AAA.

Metalloproteinases and their inhibition by TIMPs

Matrix metalloproteinases (MMPs) are proteins with a zinc-binding motif in their catalytic domain and are responsible for degrading collagens, gelatins, and elastin in the aortic wall of AAA. These proteases are involved in several biological events, such as reproduction, development, morphogenesis, and wound healing. They also play a role in protection against bacterial invasion (Shiomi, Lemaitre et al. 2010). Metalloproteinases



are zymogens and need cleavage of their pro domain to be activated. Plasmin, a protein involved in fibrinolysis, is considered to be an activator of metalloproteinases (Allaire, Hasenstab et al. 1998).

The major collagenases are MMP1, MMP8 and MMP13. These proteases are able to degrade triple helical domain of collagens at physiological pH. The major gelatinases are MMP2 and MMP9 and are capable of digesting denatured collagen.

Tissue inhibitors of metalloproteinases (TIMPs) have many biological roles, such as modulation of cell proliferation, anti-angiogenesis, anti- and pro-apoptosis, signaling, and cell migration (Brew and Nagase 2010), but the most studied function of TIMPs is the inhibition of metalloproteinases. TIMPs 1-4 inhibit MMPs (Brew and Nagase 2010; Shiomi, Lemaitre et al. 2010). In one study, Lipp et al showed that TIMPs 1-3 in human AAA are suppressed compared to control aorta (Lipp, Lohoefer et al. 2012). The elastase-induced model of AAA produces a more severe phenotype in mice lacking expression of TIMP1 (Eskandari, Vijungco et al. 2005). Because TIMPs have anti-angiogenic properties (Brew and Nagase 2010), a lack of TIMPs in AAA tissue could promote angiogenesis.

In a recent study, the thickness of the ILT was correlated with the concentration of TIMP1 and active MMP9 in the aortic wall of AAA and that showed to be independent of other variables, such as statin use, age, and gender (Khan, Abdul Rahman et al. 2012).

The rupture site of AAA shows an increase in the concentrations of MMPs, such as MMP8 and MMP9 (Choke, Thompson et al. 2006; Wilson, Anderton et al. 2006). In the elastase-induced model of AAA, mice deficient in MMP9 (*Mmp9*^{-/-}) showed attenuation of AAA progression compared to wild-type *Mmp*^{+/+} controls (Thompson, Curci et al. 2006). MMP9 activity is preserved when the protein binds neutrophil gelatinase-associated lipocalin (N-GAL), because degradation of MMP9 is halted (Fernandez, Yan et al. 2005). Thus, N-GAL enables MMP9 to continue to degrade collagen and elastin of AAA wall.

In AAA, MMPs are up-regulated, while their inhibitors, TIMPs, are suppressed.

ADAMs (A Disintegrin And Metalloproteinase) are a group of proteases belonging to the metalloproteinase family, but several members of this group have a trans-membrane domain. Similar to other MMPs, ADAMs also contain a zinc-binding domain in their active site. ADAMs are produced as zymogens and are activated in the cytoplasm before they are transported to the cell membrane, where they are anchored in the phospholipid bilayer by their trans-membrane domain (Edwards, Handsley et al. 2008).

ADAM10 and ADAM17, which are studied in this thesis, cleave other membrane proteins, causing them to become activated or shed. Tumor necrosis factor alpha (TNF α) is one of the proteins activated by ADAMs that possess TNF α converting enzyme (TACE) activity, especially ADAM10 and ADAM17 (Hiraoka, Yoshida et al. 2008).

TNF α is a cytokine produced by activated macrophages and other cells. TNF α has a major role in the induction of inflammation and apoptosis (Locksley, Killeen et al. 2001). In the elastase-induced model of AAA, mice lacking receptors for TNF α showed only limited development of AAA (Thompson, Curci et al. 2006). TNF α , together with c-JNK N-terminal kinase JNK and NonO, (a 54 kDa nuclear RNA and DNA binding protein)

inhibit the expression of prolyl-4 hydroxylase (P4H), an enzyme required for synthesis of all collagens. Inhibition of P4H decreases collagen synthesis. TNF α and JNK also participate in the induction of genes, such as MMPs, that are responsible for matrix degradation (Yoshimura, Aoki et al. 2005; Zhang, Zhang et al. 2007).

It has been shown that ADAM17 (TACE) is up-regulated in the media and adventitia of the aneurysmal wall and that TNF α has a role in the pathogenesis of AAA (Satoh, Nakamura et al. 2004).

In addition to TNF α , the substrates for ADAMs include other vessel-wall proteins, such as syndecan -1 and -4, collagen type XVII, ICAM-1 and VCAM-1 (Scheller, Chalaris et al. 2011). Inhibitors of ADAM10 and ADAM17 include TIMPs 1-3.

ADAMs are a group of specialized MMPs that have been implicated in the pathogenesis of AAA.

Cathepsins, Tryptase, Chymase, Granzyme B

Other proteases that may play a role in the pathogenesis of AAA include cathepsins, tryptase, chymase, and Granzyme B.

Cathepsins are cysteine, aspartyl or serine proteases that are active mostly in the lysosomes of cells involved in protein degradation. Twelve members of this family have been identified. They are active at low pH and have been indicated to have a role in elastolysis (Sukhova and Shi 2006; Lohoefer, Reeps et al. 2012).

In the elastase-induced model of AAA, mice deficient in cathepsin C (Shi 2007) and L (Sun, Sukhova et al. 2011) were resistant to developing aneurysms.

Active cathepsin D and cathepsin L were found in the aneurysmal wall, and their concentrations were higher in AAA than in healthy control aorta. The activity of these proteases was shown to be higher in the ILT of AAA compared to an in-vitro produced blood clot from donors (Gacko and Glowinski 1998). Shortly after, the same group showed that activity of other cathepsins, namely, cathepsin A, cathepsin D, and cathepsin G, were higher in the ILT than in the blood clot while activity of cathepsin B, cathepsin C and cathepsin E was only slightly higher in the ILT of AAA patients compared to the blood clot (Gacko and Glowinski 1998). In addition, plasma of AAA patients was reported to contain elevated concentrations and activity of cathepsin D compared to control plasma (Gacko, Guzowski et al. 2006).

Another group showed that cathepsin originates from neutrophils and mast cells in AAA tissue (Mayranpaa, Trosien et al. 2009). Mast cells also release tryptase and chymase during degranulation and are abundant in AAA. Both tryptase and chymase are involved in the activation of MMPs and cause apoptosis of smooth muscle cells in AAA (Swedenborg, Mayranpaa et al. 2011).

Granzyme B is a protease produced by cytotoxic T-lymphocytes involved in antitumor and antiviral activities of host immune defense, and their presence in AAA tissue has been demonstrated (Chamberlain, Ang et al. 2010).

Cathepsins, granzyme B, and mast cell proteases may also participate in the pathogenesis of AAA.



The Intraluminal Thrombus

Most AAAs of a size that approaches a risk of rupture contain an Intraluminal thrombus (ILT) (Hans et al.), which is a non-homogenous multilayer clot, that adheres to part or all of the dilated aortic wall. The ILT retains blood components within its fibrin mesh (Figure 4).

Low sheer stress, turbulent flow within the AAA sac, endothelial injury, and platelet activation favor formation of the ILT in AAA (Touat, Ollivier et al. 2006) (Biasetti, Gasser et al. 2010).

Distorted anatomy and vascular function of AAA provoke the formation of ILT.

Arterial thrombi and venous thrombi differ in composition owing to hemodynamic factors. Venous thrombi are formed under low flow and are composed of fibrin, a network with few platelets and relatively more red blood cells. Arterial thrombi develop under high flow and are composed of a fibrin mesh with aggregated platelets (Bouchard, Silveira et al. 2001).

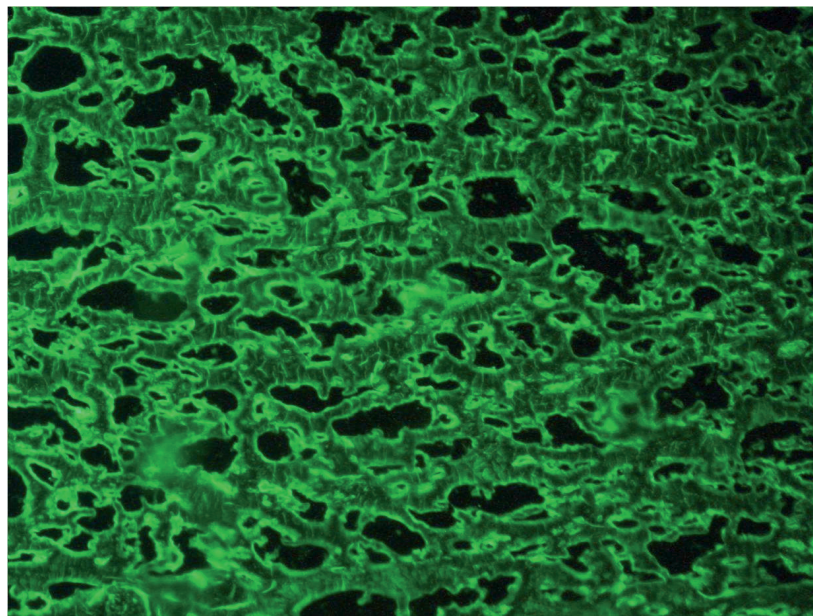


Figure 4: *Fluorescent image of fibrin-FITC immunostaining of the ILT*

Studies show that risk of rupture of AAA increase with the thickness of the ILT (Satta, Laara et al. 1996; Hans, Jareunpoon et al. 2005). One explanation is that ILT volume correlates with AAA diameter (Shindo, Matsumoto et al. 2005), and AAA diameter correlates with risk of rupture. A second explanation is that the ILT itself exerts biological effects that destabilize AAA (Choke, Cockerill et al. 2005).

Properties of the ILT

In platelet-rich arterial thrombi the fibers of polymerized fibrin are thinner and denser compared to fibrin fibers in platelet-poor venous clots (Collet, Montalescot et al. 2002). The

tightly arranged fibers in arterial thrombi restrict binding of tissue plasminogen activator (tPA) for initiation of fibrinolysis. Moreover activated platelets releasing proteins such as Myosin can mask plasmin cleavage sites of fibrin (Kolev, Lerant et al. 1994). These effects make arterial thrombi resistant to fibrinolysis. Likewise, the dense structure of arterial thrombi makes them highly resistant to mechanical forces. Consequently vast presence of platelets in arterial thrombi enables a more stable and long lasting thrombus. The ILT renews as it recruits platelets and fibrinogen from flowing blood (Touat, Ollivier et al. 2006). In this way, the layer adjacent to the aortic wall, called the abluminal layer, is considered to be the oldest. The most recently generated layer, the luminal layer, is in contact with the blood and attracts hematopoietic cells and other blood components (Houard, Touat et al. 2009). The layer in between these two layers is the middle layer, Figure 5, panel A.

In experimental animal models of aneurysms such as the elastase-induced model (Laser, Lu et al. 2012) and Angiotensin II- induced model (Daugherty and Cassis 2004) some development of ILT has been observed.

The human ILT consists of various layers with no distinctive borderlines between them (Touat, Ollivier et al. 2006). Dependent on its spatial relation to the vessel wall or blood flow, it differs in cell density and texture.

Figure 5 uses several techniques to show a typical ILT. As can be seen in this figure, an ILT is not a homogenous structure. The different layers are most evident in the gross sample (panel A). The photomicrographs in panels B-D, where the density of the cells in various layers of the ILT are seen by nucleus staining. It is evident from this image that the luminal layer is the most cell-rich, while the abluminal layer is nearly acellular. Platelets and neutrophils are abundant in the ILT, but platelets do not stain with heamatoxylin due to lack of nucleus. To characterize the content of the ILT with spatial specificity, we decided to divide the ILT into different regions. The layer closest to the aortic wall of AAA is macroscopically and microscopically different from the layers farther from the AAA wall.

Different layers of the ILT have different properties.

In calculating the strength of AAA, thickness of the ILT is used as a variable to predict rupture along with gender, age, normalized transverse diameter, family history, and current smoking (Vande Geest, Wang et al. 2006). Other studies found a correlation between volume of the ILT and AAA progression (Speelman, Schurink et al. 2010; Parr, McCann et al. 2011) and some researchers suggest that the rate of growth of the ILT is a predictor for rupture (Stenbaek, Kalin et al. 2000).

Since the abdominal region of the aorta lacks vasa vasora, the cells in the aortic wall are dependent on blood flow in the aortic lumen for survival. The presence of an ILT obstructs access of nutrient blood to the aortic wall particularly if the ILT is thick. Presence of a 3mm thick ILT prevents the passage of O₂ to the vessel wall (Vorp, Wang et al. 1998), which leads to hypoxia and concomitant neovascularization at the site of ILT-covered



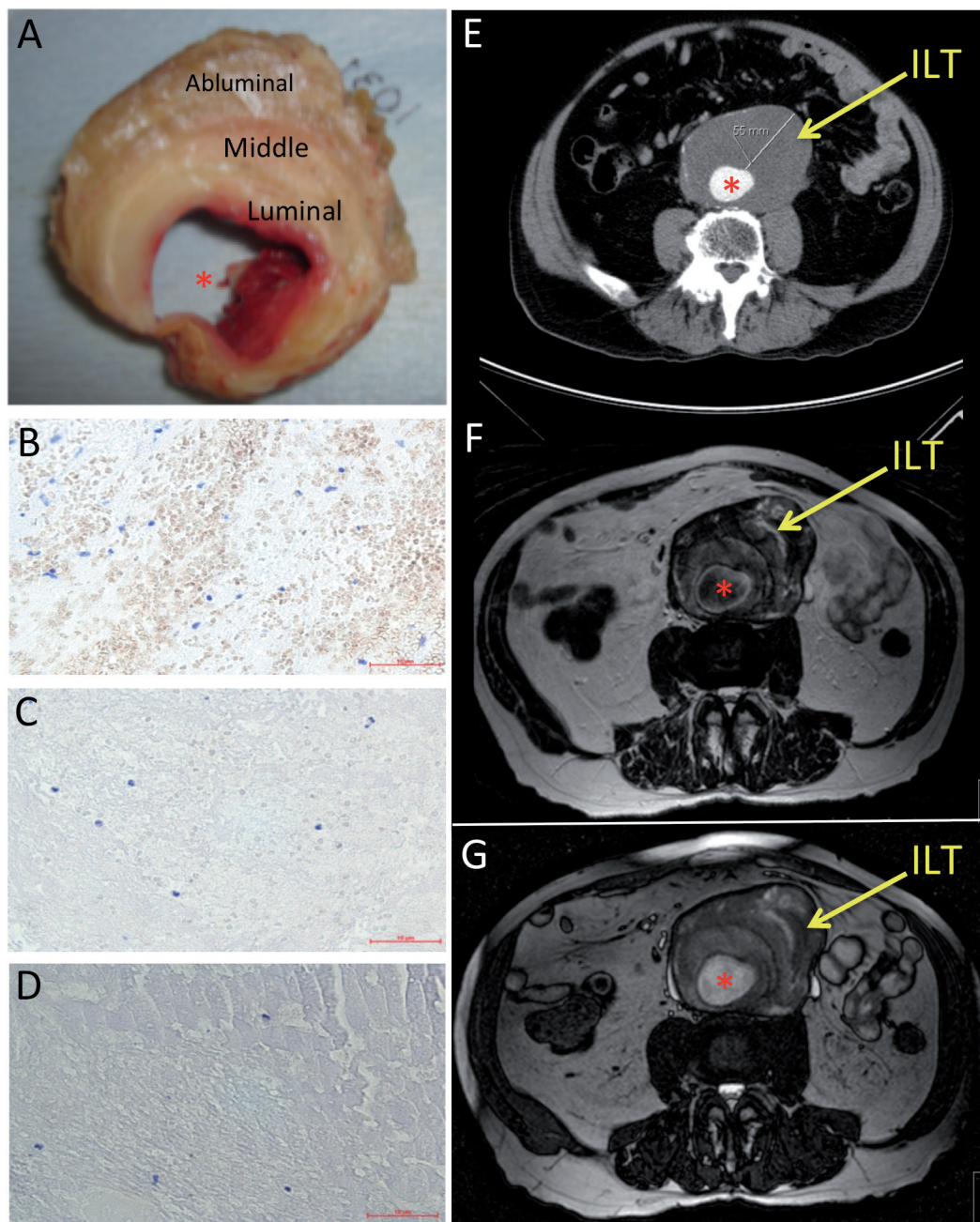


Figure 5: ILT seen using five methods: Panel A) Cross-section of a typical intraluminal thrombus obtained during open repair. The layers are depicted in the figure. The thin red part is the luminal layer and the opposite site, meaning the grayish part (that was situated adjacent to the aortic wall), is the abluminal layer, and the layer in between is the middle layer. Panels B-D) show Haematoxylin staining of each of the three layer of the same ILT. The luminal layer is the most cellular; and the abluminal layer is essentially acellular. Panel E) Contrast enhanced computed tomography of the same AAA before surgical repair. The white line indicates the thickness of the thrombus. F, G) T2 weighted turbo spin-echo (F) and Steady state free precession gradient echo (G) magnetic resonance images of the aorta before surgery at a level corresponding to the computed tomography image E. By magnetic resonance imaging, different parts of the thrombus show a greater heterogeneity than on computed tomography. The red star shows the path of blood flow in panels A, E, F and G and the yellow arrow in panels E-G show the ILT.

AAA wall (Holmes, Liao et al. 1995; Vorp, Lee et al. 2001; Choke, Thompson et al. 2006).

In some biopsies of an aortic wall covered by thick ILT, which obstructs the vessel from direct contact to the blood, neovascularization is seen (Mayranpaa, Trosien et al. 2009) (Thompson, Jones et al. 1996; Choke, Cockerill et al. 2006). The presence of new vessels facilitates entrance of inflammatory cells into the aortic wall (Reeps, Pelisek et al. 2009). Moreover, it has been postulated that stem cell factor produced by endothelium from newly formed vessels in AAA wall attracts mast cells and platelets to this environment (Mayranpaa, Trosien et al. 2009). Figure 3 above shows a cross section of an aneurysm wall. In this Figure the arrow indicates a site of neovascularization.

Presence of the ILT causes hypoxia, which leads to neovascularization

Cells and proteases in the ILT

Thompson et al showed that neovascularization can enhance the recruitment of inflammatory cells into the aortic wall (Thompson, Jones et al. 1996) and these cells can express and release MMPs within the weakened AAA wall (Sakalihasan, Delvenne et al. 1996). Moreover, MMPs are capable of enhancing the activity of other proteases (Carrell, Burnand et al. 2006). It has also been shown that the ILT has the ability to trap proteases, such as MMP2 and MMP9 (Fontaine, Jacob et al. 2002) and inflammatory cells such as neutrophils are readily recruited into the luminal layer of the ILT (Houard, Touat et al. 2009). Additionally, the cell population in the ILT mostly consists of neutrophils. Platelets are also present in all layers of the ILT, but presence of macrophages, T cells and B cells have not been widely observed within the ILT.

Several cell types besides platelets are trapped within the fibrin network of the ILT. The consensus is that hematopoietic cells are generally trapped in the ILT (Sagan, Mrowiecki et al. 2012). However, CD3-positive cells (T cells), CD45-positive cells and hematopoietic cells except erythrocytes and platelets are not readily observed in the ILT. In contrast, T and B cells are found in the aneurysm wall (Ocana, Bohorquez et al. 2003). Other cells present in the ILT are red blood cells, which are found primarily in the luminal layer of the ILT with some in the middle and essentially none in the abluminal layer.

The predominant cell types trapped within the ILT are neutrophils and platelets, chiefly in the luminal layer.

Neutrophils are well known to express several proteases. In addition, platelets are capable of expressing MMP1, MMP2 and MMP9 (Leza, Salas et al. 1998; Fernandez-Patron, Martinez-Cuesta et al. 1999; Galt, Lindemann et al. 2002). During clot formation, platelets directly recruit neutrophils. Activation causes platelets to display P-selectin and $\beta 2$ and $\beta 3$ integrin on their surface, and these molecules bind counter-receptors on neutrophils. Platelets also induce neutrophils to produce cytokines and release granules that contain proteases, such as elastase and MMPs (McEver, Moore et al. 1995). Neutrophils possess three types of granules: primary, secondary and tertiary. Primary granules are identified by the presence of myeloperoxidase, and they also contain serine proteases, such as elastase, cathepsin G and proteinase 3. Secondary granules contain zinc-dependent collagenases,



while gelatinases MMP2 and MMP9 are held in tertiary granules. These proteases are capable of degrading the vessel wall matrix. Nevertheless, the presence and activity of these proteases within the various layers of the ILT have not been demonstrated. Red blood cells have been observed in the luminal layer of the ILT, but the role of these cells in pathogenesis of AAA have not been studied.

ILT traps cells that produce proteases that may weaken the aortic wall, promoting rupture.

Effect of the ILT

The presence of an ILT may partially compensate for the hemodynamic effects from arterial expansion. An ILT can restore the lumen of a distended aorta to a semi-normal size and to a certain extent absorbs the stress on the degraded AAA like a cushion, thereby lowering wall stress (Speelman, Schurink et al. 2010), in other words, ILT supports the redistribution of the stress on the wall (Di Martino, Mantero et al. 1998; Hinnen, Visser et al. 2005). This effect is important as long as the ILT is intact, but it has been shown that small fractures in the surface of the ILT can propagate to the wall of the aneurysm, concentrating the stress of the focal areas leading to rupture (Gasser, Gorgulu et al. 2008).

The presence of an ILT may have both protective and harmful consequences.

The wall under the ILT (Thrombus vessel wall, TVW) and the thrombus-free wall (non-thrombus vessel wall, NTVW) exhibit important differences. Figure 6 shows a CT image from an AAA patient in whom an eccentric ILT covers part of the aneurysm wall, while NTVW remains in contact with the blood flow. In our previous studies we showed that the wall under the ILT is thinner and prone to rupture compared to NTVW (Kazi, Thyberg et al. 2003). One study of ruptured aneurysm indicates that 80% of ruptures occur under the ILT (Simao da Silva, Rodrigues et al. 2000).

The difference between TVW versus NTVW was further investigated by evaluating computed tomography (CT) images from ruptured AAAs just before emergency surgical repair. In many cases, a crescent sign appears in the ILT, indicating bleeding into the ILT associated with rupture (Arita, Matsunaga et al. 1997). Roy et al showed that 52% of the ruptures occur at the site where the AAA is covered by the ILT, and 14% occur at the edges of the ILT, while only 34% occur at the site that according to CT images appear to be free from ILT (Roy, Labruto et al. 2008).

Most ruptures occur in the weakened aortic wall under the ILT.

Prior studies of AAA have largely focused on the dilated, damaged aortic wall. Here, we focused on the ILT, in order to understand its possible role in the pathogenesis and rupture of AAA. We hypothesized that the ILT contains cells that release proteases that damage the immediately underlying section of the aortic wall predisposing to rupture. Moreover, the abluminal layer, which contacts the underlying aortic wall, is nearly acellular. Thus,

the major goals of this work using human material was to determine if the ILT contains proteases, which are enzymatically active, and if enzymatically active proteases are present specifically within the abluminal layer, next to the dilated aortic wall.

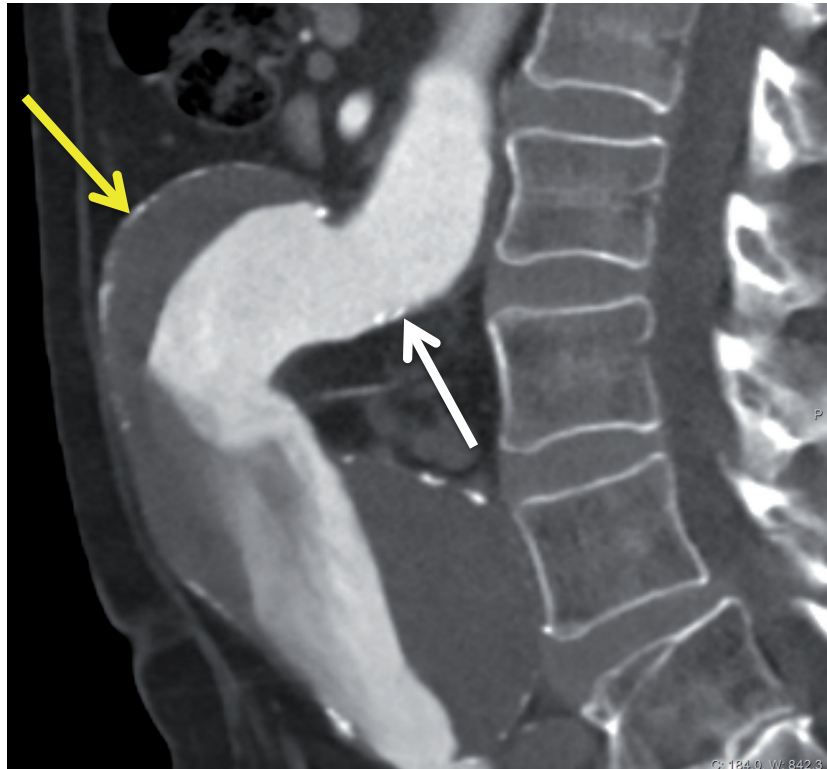


Figure 6: A Computed Tomography (CT) image from an AAA patient. The image shows sagittal section, in which the patient's anterior abdomen is on the left, and the vertebrate and the vertebral spine on the right. The white winding structure in the image is the blood flow and the yellow arrow indicates the arterial wall under the grey mass of the intraluminal thrombus (thrombus vessel wall TVW) while the white arrow indicates a nearby section of aortic wall that is in direct contact with blood flow (non-thrombus vessel wall NTVW).



AIM OF THIS THESIS

Investigating the components of the intraluminal thrombus (ILT) that could influence abdominal aortic aneurysm (AAA) pathogenesis.

Paper 1: To analyze the presence of N-GAL in AAA tissue which could enhance or preserve MMP9 activity

Paper 2: To find evidence that ILT contains active collagenases and gelatinases

Paper 3: To investigate whether the abluminal layer of ILT that is lacking cells can harbor microvesicles carrying membrane bound proteases.

Supplementary Paper: To investigate mechanisms and possible importance for fibrinolysis of ILT by Neutrophil elastase.



MATERIALS AND METHODS

Patients

From 2006-2012, we recruited patients from the department of Vascular Surgery at the Karolinska Hospital. Inclusion criteria for our study were elective open repair of abdominal aortic aneurysm (AAA) ≥ 55 mm in diameter, and willingness to participate in our study. Exclusion criteria were endovascular repair, dissection, and inflammatory aneurysm. Currently, there is clinical equipoise between open versus endovascular repair, but open surgery involves removal of intraluminal thrombus (ILT) as part of the procedure. The study and informed consent were approved by the Regional Ethics Committee.

Before July 2010, patients with AAA were typically identified by chance after CT, MRI, or ultrasound examinations of the abdomen for other reasons. In July 2010, ultrasound screening for AAA was introduced in Stockholm County for all men 65 years of age, and patients with AAA exceeding 55 mm in diameter were offered elective surgery. We collected plasma samples and biopsies of aorta and intraluminal thrombus (ILT) from a total of 57 patients (14 women and 43 men, average age 71, all present or previous smokers except for one man during the five years of the study).

In open surgical repair of AAA, the abdomen is opened and the aorta is clamped from above, distal to the renal arteries, and distally to the aneurysmal widening, whether this reaches the distal aorta or the iliac arteries (Figure 1).



Figure 1: Clamping of the abdominal aorta in a case where widening of the iliac arteries is not present.

The enlarged aorta is opened longitudinally and the aneurysmal section of the aorta is removed and replaced by a synthetic graft. In most cases, an ILT is present, and removed as well (Figure 2). Sometimes, a fluid in the region between ILT and the underlying wall can be obtained; when possible, we collected that liquid as well, which we termed “interface fluid”.

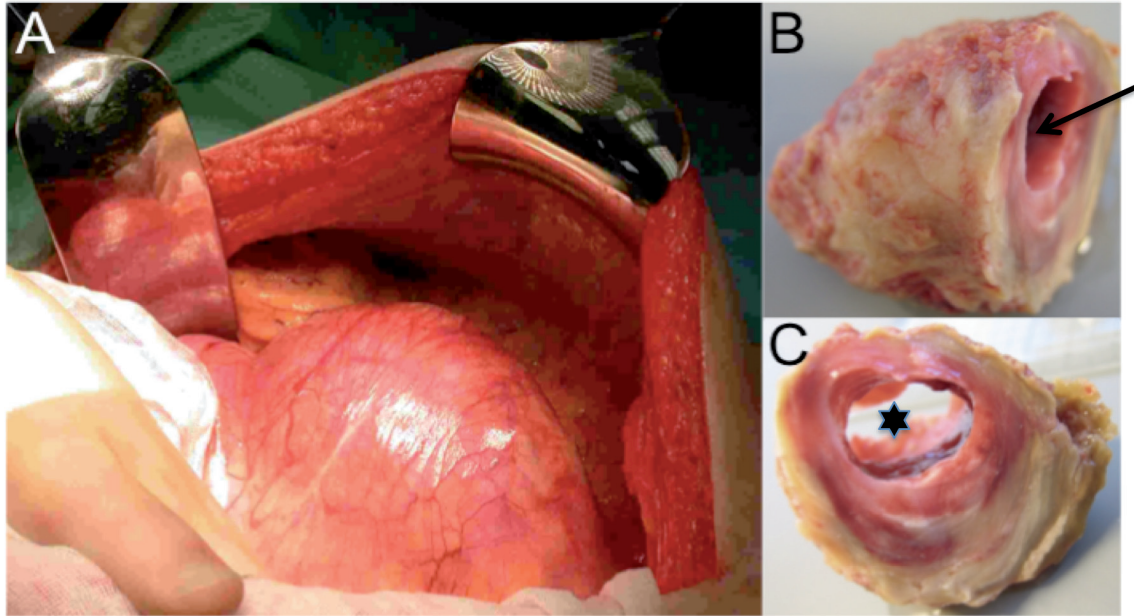


Figure 2: A-The bulging aorta is seen here, viewed from the outside, after opening the abdomen but before clamping and opening the aneurysm. Panels B and C show images of an ILT. The arrow (B) and asterisk (C) mark the passage for the flow of blood (B, C).

Samples collected

Blood samples anti-coagulated with either EDTA or citrate was obtained before surgery. Since platelets can carry proteases of interest in our study, we decided to prepare platelet free plasma by two centrifugations. Blood was centrifuged at 2500 g for 20 minutes at room temperature in order to remove red and white blood cells, and then the supernatant was centrifuged at 20,000 g for 30 minutes at 4°C to pellet platelets. Platelet-free supernatants were harvested, aliquoted, and stored at -80°C until use. Interface fluid, when available, was also aliquoted and stored at -80°C until use.

The aortic biopsies that we collected were from 2 different regions of the aneurysm – namely, a portion of the enlarged aorta lying directly under the ILT (called thrombus vessel wall, TVW) and a portion of aorta which was dilated but not in direct contact with the intra luminal thrombus, as identified by pre-operative CT-image (called non-thrombus vessel wall, NTVW). For clarity see the CT-image in Figure 3. The vessel biopsies were prepared for RNA and protein extraction and also for immunohistochemistry (IHC), as described below.



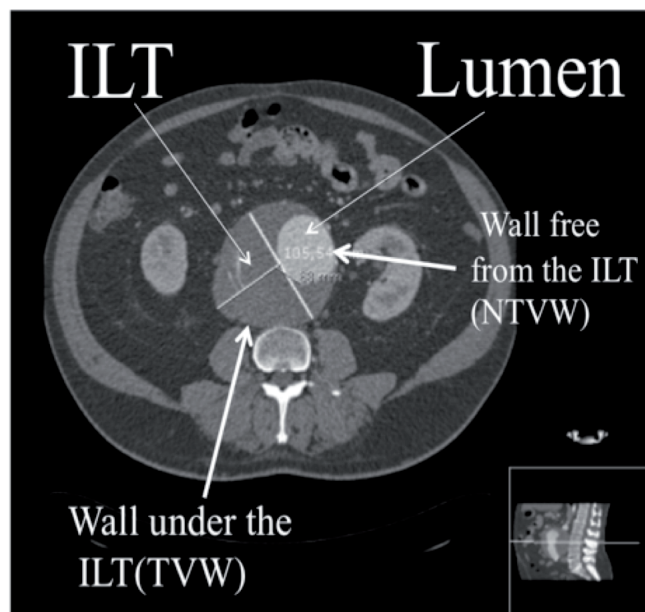


Figure 3: Computed tomography (CT) image from a large abdominal aortic aneurysm. This image is a cross section of abdomen of the patient. In this image, the ILT can be seen covering one side of the aneurysm while allowing the other part of the aneurysm wall to be in contact with the blood flow.

For the ILT, we received the entire thrombus from surgery. At the laboratory, we divided each ILT into three macroscopically evident layers. The layer closest to the blood stream, the luminal layer, is red and to some extent elastic. The layer closest to the vessel wall, the abluminal layer, is a layer with the most degraded texture, appears grey and easily falls apart. The intermediate layer in the middle of these two was called the middle layer

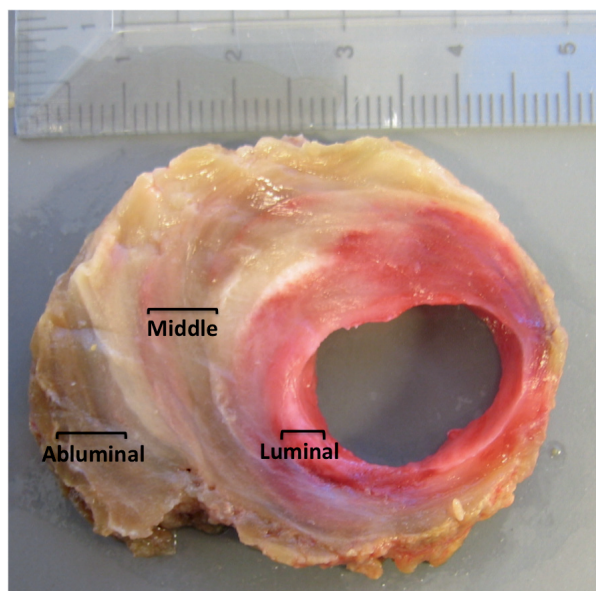


Figure 4: A typical Intra Luminal Thrombus (ILT) where the luminal, middle and abluminal regions of the ILT are macroscopically evident. Scale unit is centimeters.

Most ILTs collected had these distinct characteristics. The ILTs that differed had non-distinguishable layers. These ILTs were not included in the study. The samples of ILT

that we collected were divided for various purposes. Parts of the samples were frozen for protein studies and part was fixed with zinc formaldehyde and dehydrated and embedded in paraffin for IHC. The 3 layers of the ILT were separately processed for protein extraction and IHC.

Protein extraction

ILT and vessel biopsies obtained at surgery were snap frozen in dry ice. Frozen pieces (0.1-0.6 g) were thawed later and cut into small pieces, placed in 2 ml tubes containing small (1.4mm in diameter) porcelain spheres and 1.0 ml RIPA buffer at pH 8. Homogenization was performed with a PhastPrep machine (FastPrep FP120 Homogenizer, BIO101/FP120, Lab Recyclers, Gaithersburg, MD) with mechanical agitations of 20 seconds separated by 2-min cooling periods resulting in a homogenous suspension. Protein quantification kit (Thermo scientific, Rockford, IL, USA) was used to determine the total concentration of all protein extracts.

We also tried to extract protein using the older method of Urea-extraction. This method is robust but tedious. A concentration determination of the total protein amount from both methods showed that the difference is negligible. Thus we continued with the method described earlier.

Enzyme-linked immunosorbent assay (ELISA)

This method enables quantification of the concentrations of specific proteins. Because of our interest in collagenases and gelatinases, we purchased ELISA kits to measure MMP1, MMP2, MMP9, MMP13, Neutrophil Elastase (Paper II), N-GAL/MMP9 (Paper I), and assayed ILT protein extraction and plasma of the patients. Peptides from degradation of fibrin by elastase (XDP) were measured with a specific monoclonal antibody anti-elastase-XDP clone IF-123 from Cosmo Bio Co. Ltd., (Tokyo, Japan) in sandwich ELISA assay developed in our laboratory (ongoing project).

Proteases that were present in the AAA tissue with high concentrations were further analyzed. MMP9, Neutrophil elastase (Paper II) and ADAM17 (Paper III) were also analyzed for their enzymatic activities.

Real time reverse-transcriptase quantitative PCR (qPCR)

This method enables us to quantify levels of specific mRNA expressed in tissues. For this analysis, we obtained vessel wall biopsies from AAA patients, both TVW and NTVW.

Aortic tissue samples were fixed in RNA-Later (Qiagen, Sweden) at 4° C for 24 hours and stored at -80°C before RNA extraction using the RNA-extraction kit from Qiagen. We also tried to extract RNA from all layers of the ILT but the amount of RNA and its quality was not sufficient for further analysis, consistent with the relatively low numbers of cells, particularly in the abluminal layer. In paper III the aortic tissue was divided to media and adventitia for better understanding the cell types that express ADAM10 and ADAM17.

Quality and quantity of RNA were analyzed by an Agilent 2100 Bio analyzer (Agilent



Technologies, Paolo Alto, CA, USA). cDNA was produced from mRNA and real time PCR assay was performed. Primers for *N-GAL* (Paper 1), *ADAM10* and *ADAM17* (paper 3) Ribosomal protein large P0 (RPLP0) was used as a housekeeping gene for normalization. Samples were run on a (Bio-Rad, DNA engine, Tetrad2, Peltier thermal cycler), and data analysis was performed using the manufacturer's software Applied Biosystem, "7000 system software" version 1.2.3. and Excel-based software packages for determining the values and Statistica software for the statistic.

Co-immunoprecipitations, and zymography

In Paper I, we were interested to find out whether N-GAL in AAA tissue is physically associated with MMP9. Therefore, protein extracted from ILT and the aortic wall of AAA was immunoprecipitated with an antibody against N-GAL, followed by immunoblotting for MMP9. Agarose beads are used to bind antibody against the protein of interest. Adding the antibody-bead to the protein suspension allows the protein of interest to pellet after a centrifugation step. Pellet will include protein of interest and its physically associated proteins. An immunoblot in reducing condition will facilitate the disassociating of bonds to investigate single proteins further.

Investigating the activity of gelatinases such as MMP2 and MMP9 is possible by Zymography. To perform this method, an electrophoresis zymogram gelatin gel was used. These gels and buffers needed were purchased from Bio-Rad (Stockholm, Sweden).

Once the samples were loaded to the vertical zymogram, we electrophoresed at 100 volts for 3-4 hours. After electrophoresis, gels were incubated with a renaturing buffer that allows the MMPs to undertake their original structure and become activated. The gel was then incubated in developing buffer overnight at 37 °C to allow digestion of the gelatin in the gel to occur. Then the gel was stained with coomassie blue for 10 min followed by several washes. Clear bands in a dark blue background emerges after 3rd -4th washes. These bright bands are a result of activity of MMPs in the sample. An electrophoresis marker is used as a reference to estimate the weight of the proteases that degraded the gel on different lanes. These bands will appear at the estimated weight for pro- MMPs and active MMPs where the difference is a few kDa. (Pro-MMP9 92 kDa and active MMP9 83 kDa, Pro-MMP2 is 72 kDa and its active form is 62 kDa).

Immunohistochemistry (IHC)

The presence and distribution of specific cell types and proteins in tissue sections can be observed with IHC. In this method, antibodies against a cell marker or protein of interest are used to stain the sections. The ILT and vessel biopsies from surgery of AAA patients were fixed in Zinc formaldehyde (Histolab, Göteborg, Sweden), with a 20:1 volume ratio to the tissue for a proper fixation. After 24 hours of fixation the tissue was kept in 70% ethanol until further dehydration. Tissues were then dehydrated using a VIP dehydration machine (VIP 2000B, SAKURA, USA). The specimens were then embedded in paraffin and sectioned at 5µm thickness. The sections were rehydrated followed by an antigen retrieval step, using DIVA buffer and background blocking using Sniper. After this step the sections were incubated with primary antibodies, one hour in room temperature

followed by secondary antibodies, then stained using the chromogens DAB or Vulcan fast red which are stable chromogens or Frangi blue that is less stable. All the reagents belong to MACH II and III systems (Biocare Medical, Concord, CA, USA).

We focused on cells and proteins in the ILT and vessel wall of AAA. In Paper I, we used antibodies specific for, neutrophils (anti-CD66acd), MMP9, and N-GAL. In Paper II we stained ILT sections with markers for macrophages, (CD163), neutrophils (CD66acd), mast cells (anti tryptase), and platelets (anti-CD61), although in this paper, figure 2 states CD161, which is incorrect. In Paper III, we examined the membrane bound proteases ADAM10 and ADAM17 in the ILT and AAA tissue.

Transmission electron microscopy (TEM)

This method allows monitoring small components of tissue down to sizes around 100nm in diameter. The method was used in Paper III, where the presence of membrane microvesicles (MVs) was verified. To perform immunostaining by TEM, the secondary antibody is attached to a small gold particle, which appears as a solid, electrodense circle in the images. In the case of double staining, the sizes of those particles (5 and 10 nm) differ and allow the distinction between different antibodies.

Fresh ILT and AAA vessel wall were collected from surgery and fixed in 3% paraformaldehyde in 0.1 M phosphate buffer and infiltrated into 2.3 M of sucrose. The further preparation of samples for EM microscopy was carried out at the Electron Microscopy Core Facility of Karolinska Institutet. See the procedure from this facility below.

Immunoelectron microscopy (iEM)

Samples were fixed in 3 % paraformaldehyde in 0.1 M phosphate buffer and infiltrated into 2.3 M sucrose and frozen in liquid nitrogen. Sectioning was performed at -95°C and placed on carbon-reinforced formvar-coated, one hole Nickel grids. Immunolabelling procedure was performed as follows: grids were placed directly on drops of 0.15 M sodium cacodylate (caco) buffer containing 2% BSA (Sigma fraction V) and 2% Fish gelatin (GE Healthcare, Buckinghamshire, UK). Sections were then incubated with the primary antibodies ADAM 10 (goat) + CD61acd (mouse) and ADAM 17 (mouse) + CD 66 (rat) in 0.15M of caco containing 0.1% BSA + 0.1% Gelatin (CBG) over night in a humidified chamber in refrigerator. The sections were thoroughly washed in the same buffer and bound antibodies were detected with Goatamouse (5nm gold) + Rabbitagoat (10nm gold) respectively Goatamouse (5nm gold) + Goatarat (10 nm gold) (Biocell, BBInternational, Cardiff, England) at a final dilution of 1:100. Sections were rinsed in 0,15M buffer and fixed in 2% glutaraldehyde and contrasted with 0,05% uranyl acetate and embedded in 1% methylcellulose and examined in a Tecnai G2 Bio TWIN (FEI company, Eindhoven, The Netherlands) at 100 kV. Digital images were taken by a Veleta camera (Soft Imaging System GmbH, Münster, Germany) (Qinyang et al, 2004)

Qinyang, W., Hultenby, K., Elhassan, A., Lindgren, U. Galanin in adjuvant arthritis in the rat *J Rheumatology* 31:2, 302-307, 2004



Flow cytometry

In Paper III, we used this method to analyze fresh tissue extracts from the ILT, for membrane-bound proteases carried on small particles such as MVs. Human AAA tissue, ILT and vessel wall samples were collected from surgery. Freshly collected biopsies were first rinsed 3 times in PBS, that we had filtered to remove any particulate matter that would produce artificial signals on flow cytometry. The rinsed tissue samples were then minced into small pieces in filtered, phenol-red-free MEM medium with 0.2% BSA (200 mg tissue per ml of MEM) and fixed 1h in 1% paraformaldehyde (PFA), followed by low-speed centrifugation at 1000 X g for 15 minutes at 4°C to remove the tissue chunks and debris. Supernatants were then centrifuged at 12600 X g for 5 minutes to remove any potential debris residues. Finally, these supernatants were divided into aliquots that were stored in freezer (-80°C) tubes (screw-tops with air-tight O-rings) for later analysis by flow cytometry.

We have tested these samples for presence of ADAM10 and ADAM17 and Neutrophil cell marker (CD66). For each antibody we used isotype control, as negative control. We also showed presence of MVs by using AnnexinV. This protein binds to Phosphatidylserine on membrane of MVs. However, activated platelets also express Phosphatidylserine but we gated for size (200nm-1µm in diameter) and by that eliminated platelets (2–3 µm in diameter). Latex beads were used as a reference for MV counts.

Immunoblots

This method shows presence and molecular weight of the protein of interest in tissue extracts. We used this method in Paper I to demonstrate the presence of MMP9 in a complex with N-GAL. In Paper II, immunoblotting was used to show the presence of MMP9 in complex with TIMP1, which inhibits MMP9 in a 1:1 molar ratio. In Paper III, immunoblotting showed the presence of ADAM10 and ADAM17 in lysates from neutrophil cell lines treated with tobacco smoke extract.

Cell-culture studies

Smoking is the leading environmental risk factor for AAA. Almost all AAA patients are or have been smokers, including the ones in this study. In Paper III, we studied the effect of tobacco smoke extract (TSE) on cultured cells by measuring their production of proteolytically active MVs in vitro. Because we identified neutrophil MVs in human ILT, we studied the human neutrophil cell line HL-60. Tobacco smoke extract (TSE, 100%) was prepared by extracting mainstream smoke from four cigarettes through culture medium. For dose response studies, cells were assessed after 24 hours of exposure and then harvested simultaneously. At the end of these treatments, cells were assessed for ADAM10 and ADAM17 expression by immunoblotting. Microvesicles were pelleted from medium by ultracentrifugation at 100,000 g and their content of ADAM proteases was also assayed by immunoblot.

Statistics

Data were analyzed using Statistica and Prism software. We used non-parametric Wilcoxon and Spearman tests, because the data sets were not normally distributed.



RESULTS AND DISCUSSIONS

The overall aim of this thesis was to investigate the role and significance of the ILT in the pathogenesis of human AAA. The majority of AAAs with a diameter indicating risk of rupture contain an ILT, and the vessel wall under the ILT is disrupted and thinner compared to the AAA vessel wall that is not situated under an ILT. Therefore, investigating specific molecular constituents of the ILT that could participate in the degradation of the AAA wall became our overall focus. We assessed the presence and enzymatic activities of a series of soluble and transmembrane proteases in different layers of the ILT and within the AAA wall.

Paper I

(investigating the presence of MMP9 complexes with N-GAL in the ILT and AAA wall)

Why study MMP9?

Matrix metalloproteinase 9 (MMP9) is a gelatinase (Sakalihasan, Delvenne et al. 1996; Wilson, Anderton et al. 2008) that has been suggested to be a major matrix degrading enzyme in AAA. Active MMP9 is capable of degrading components of extracellular matrix, such as elastin and moderately damaged or denatured collagen. Lack of intact elastin and degradation of collagen are hallmarks of AAA. In this paper, we concentrated on MMP9 and its association to neutrophil gelatinase associated lipocalin (N-GAL), a small protein that complexes with MMP9 to sustain the gelatinase activity of MMP9 (Fernandez, Yan et al. 2005). Expression of MMP9 and N-GAL has been shown to depend on c-JNK N-terminal kinase (JNK), a mitogen-activated protein kinase. Furthermore, an inhibitor of JNK, (SP600125), reduced the amount of the N-GAL/MMP9 complex in ex-vivo and cell-culture experiments (Yoshimura, Aoki et al. 2005). We hypothesized that N-GAL might be present in AAA wall and thereby increase the activity of MMP9.

Presence of N-GAL in AAA tissue

As an initial step, we assessed the presence of N-GAL in AAA tissue samples. Immunostainings of AAA vessel wall provided our first evidence for the presence of N-GAL. In the thrombus vessel wall, N-GAL was detected mainly in the interface between the thrombus and the underlying wall, although some staining could be detected within the wall itself. Furthermore, N-GAL staining was diffuse, not associated with cells in the thrombus, indicating that it had been secreted or otherwise released. Cells-associated N-GAL was seen in the medial layer of the non-thrombus vessel wall (see Origin of N-GAL, below). We then performed quantitative real-time reverse transcriptase PCR (qPCR) mRNA in the thrombus and non-thrombus AAA wall. Levels of N-GAL mRNA were low and did not show any significant difference between these wall segments (mean

Ct of 38 ± 2 , mean \pm SD). These results indicated that N-GAL protein is present in the AAA wall, but mRNA levels were low, consistent with little ongoing local synthesis.

Origin of N-GAL

As the name N-GAL implies, neutrophils were originally identified as the main source of N-GAL. Nevertheless, it has recently been shown that both monocyte-derived macrophages and smooth muscle cells have the ability to produce N-GAL (Bu, Hemdahl et al. 2006; Hemdahl, Gabrielsen et al. 2006). In our study, staining of consecutive tissue sections, as well as double stainings, showed that N-GAL expression in the AAA wall co-localizes with areas containing cells expressing CD66b, a neutrophil marker. This finding could explain the low N-GAL mRNA levels found in the vessel wall, since neutrophils have a much shorter life span than their secreted proteins.

Association of N-GAL with MMP9

The central objective of this study was a potential association between N-GAL and MMP9 in AAA tissues. Here, we continued with immunoblotting under non-reducing conditions. The non-reducing condition allows protein complexes to remain intact. To visualize the composition of the complex, the membranes were incubated with antibodies against both MMP9 and N-GAL in a consecutive order with a stripping step in between.

This analysis revealed that N-GAL/MMP9 complexes were present in both vessel walls under ILT and free from the ILT and also within the ILT. Larger complexes than the expected 1:1 associations were also identified (135 kDa and >250 kDa). Whether these complexes contained additional proteins is not known.

At a later stage, we attempted to investigate this composition by mass spectrometry to determine the constituents of these complexes. The band, however, included various proteins that overshadowed the protein complex of interest, so that a clear distinction was not possible.

We further investigated the association between N-GAL and MMP9 using several additional methods. We used immunohistochemistry to double stain AAA tissue with antibodies against N-GAL and MMP9. Their association was seen in the images as co-stained spots (Figure 1).

Next, we used co-immuno-precipitation to resolve the composition of the complex. We used an antibody against N-GAL to capture the N-GAL protein in protein extracts of AAA tissue. After centrifugation to obtain the immuno-precipitated pellet, we performed immunoblots under reducing conditions to detect MMP9 protein. Importantly, we found that the MMP9 protein that co-immuno-precipitated with N-GAL was in its active, mature form, i.e., with a molecular weight of 83 kDa, indicating that the pro-peptide had been cleaved off. Mature MMP9 in a complex with N-GAL was observed within the aneurysm wall, the interface fluid, and all three layers of the ILT (Figure 2).



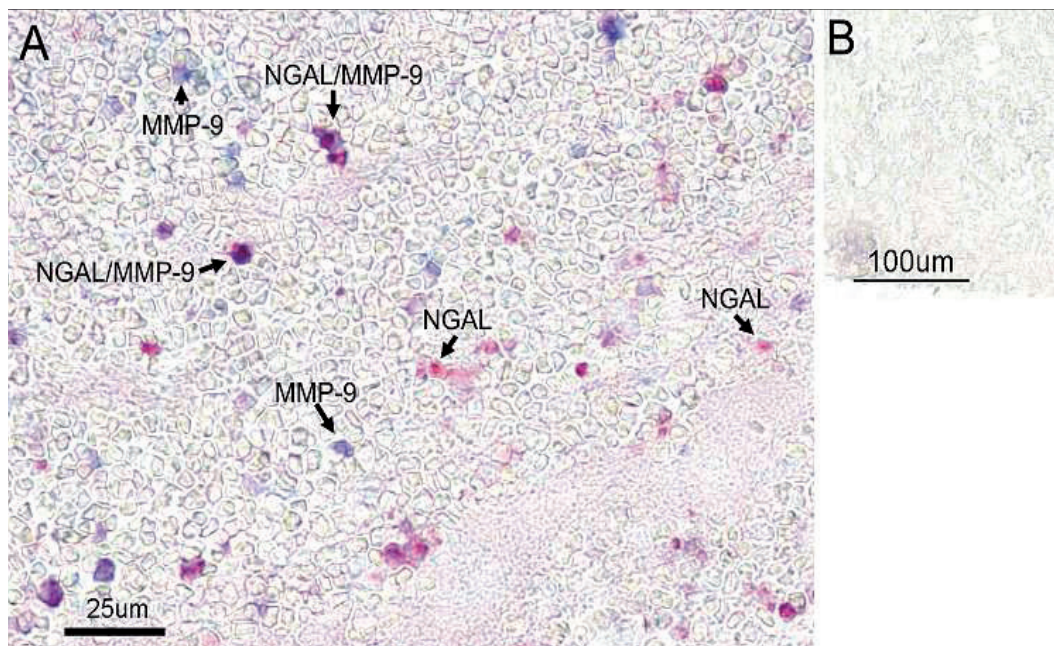


Figure 1: MMP9/N-GAL are present in the luminal layer of ILT.

A) N-GAL staining is shown in red and MMP-9 is blue, no counterstaining

B) Negative control



Figure 2: Immunoblots of MMP9 in human AAA samples.

Lane 1 shows purified mature MMP9, lane 2, purified N-GAL/MMP9 complex.

Abbreviations of AAA tissues here; interface fluid (IF), abluminal (Abl) luminal (lum) non thrombus vessel wall (NTh).

After showing that active MMP9 is in complex with N-GAL, we continued to measure the concentration of the N-GAL/MMP9 complex in AAA tissue using a more quantitative method. To do so we used an ELISA kit that specifically recognizes this complex. We measured the concentration of N-GAL/MMP9 complex in all layers of the ILT, and AAA tissue under the ILT (Th) and free from ILT (NTh) as well as in the interface fluid between the ILT and the AAA wall. Irrespective of whether we divided the original value of the protein with total protein amount or wet weight of the tissue, or both, the results showed that the concentration of N-GAL/MMP9 complex is highest in the luminal layer of the ILT, and considerably lower in the rest of the ILT (Figure 3).

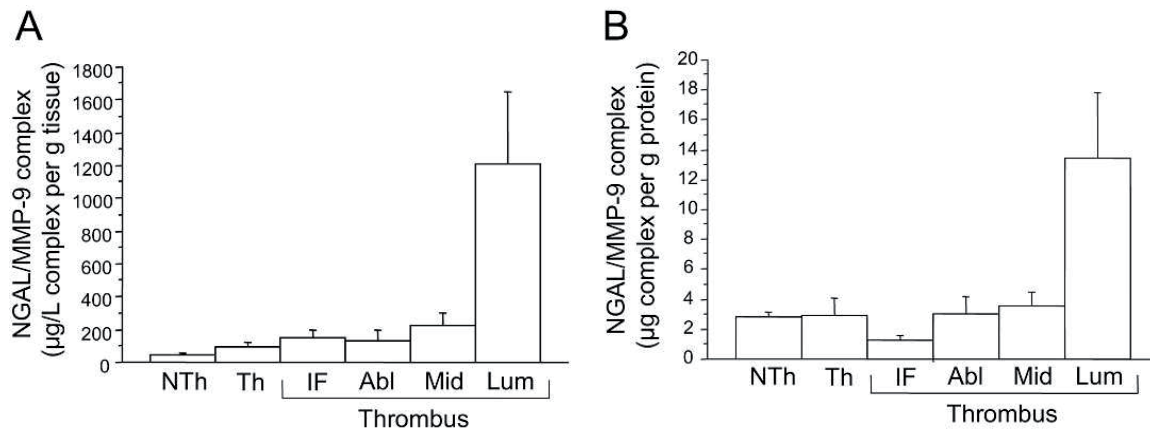


Figure 3: Concentration of N-GAL/MMP9 complex measured in AAA tissue by ELISA. A) Values normalized to the total protein in each sample, **B)** values normalized to the wet weight of the tissue before homogenization. (In this paper the term Th and NTh was used for thrombus and non thrombus vessel wall. In later papers we used TVW and NTVW denoting these walls).

This distribution presumably reflects the large accumulation of neutrophils in the luminal region of the ILT. The luminal layer is the most recently built layer of the ILT and is in constant contact with the blood flow, therefore we postulate that this layer has the ability to trap hematopoietic cells, in particular neutrophils, that might produce N-GAL/MMP9. Immunostaining showed that neutrophils present in AAA tissue are the major producers of N-GAL. Nevertheless there were also detectable amounts of N-GAL/MMP9 complexes in the abluminal layer, although much lower than that in the luminal layer of the ILT and in the underlying aneurysm wall that could have implications for degradation of the extracellular matrix during aneurysm formation.

MMP9 enzymatic activity

The association of N-GAL with MMP9 can preserve the enzymatic activity of MMP9. To analyze the activity of MMP9, we used zymography. The zymography method is based on incorporation of a protease substrate, in this case gelatin, into the electrophoresis gel. Degradation of the substrate can then be visualized as white bands in a dark blue background after Coomassie blue staining. The zymography showed that the ILT contains active MMP9. The pro form of MMP9 also appears as an active enzyme, owing to unmasking of the active site during the denaturation/renaturation procedure in this method. This method is also able to detect the presence of pro and active MMP2 (Pro-MMP9 appears at 92 kDa and active MMP9 at 83 kDa, Pro-MMP2 is 72 kDa and its active form is 62 kDa).

Take-home message

Taken together, these results show presence of enzymatically active MMP9 in complexes with N-GAL in aneurysmal tissue, but mostly in luminal layer of the ILT, away from the AAA vessel wall. These findings led us to examine other candidate proteases alongside MMP9 in papers II and III.



Paper II

(presence of soluble proteases in the ILT)

To continue our efforts to explain the degradation of the AAA wall situated under the ILT, we investigated the ILT in more detail in paper II. We analyzed the amounts of several soluble gelatinases and collagenases in the ILT, since the luminal layer of the ILT seems to be a dynamic tissue containing large amounts of proteins.

Collagenases and gelatinases in the AAA tissue

Elastin and collagen, the main matrix proteins in the aorta, are degraded by neutrophil elastase (NE) and also MMP2 and MMP9 (Swedenborg and Eriksson 2006). We measured concentrations of elastase and various collagenases and gelatinases in three different layers of the ILT, the luminal layer facing the blood flow, the abluminal layer adjacent to the vessel wall and the middle layer in between the other two layers.

Composition of a thin ILT

Dividing the ILT into three layers is only possible if the ILT is thick enough. The composition of a thin ILT (<10 mm) is comparable to the luminal layer of the thick ILT. Similar to the luminal layer of a thick ILT, a thin ILT is red and contains a large number of Neutrophils (Figure 4).

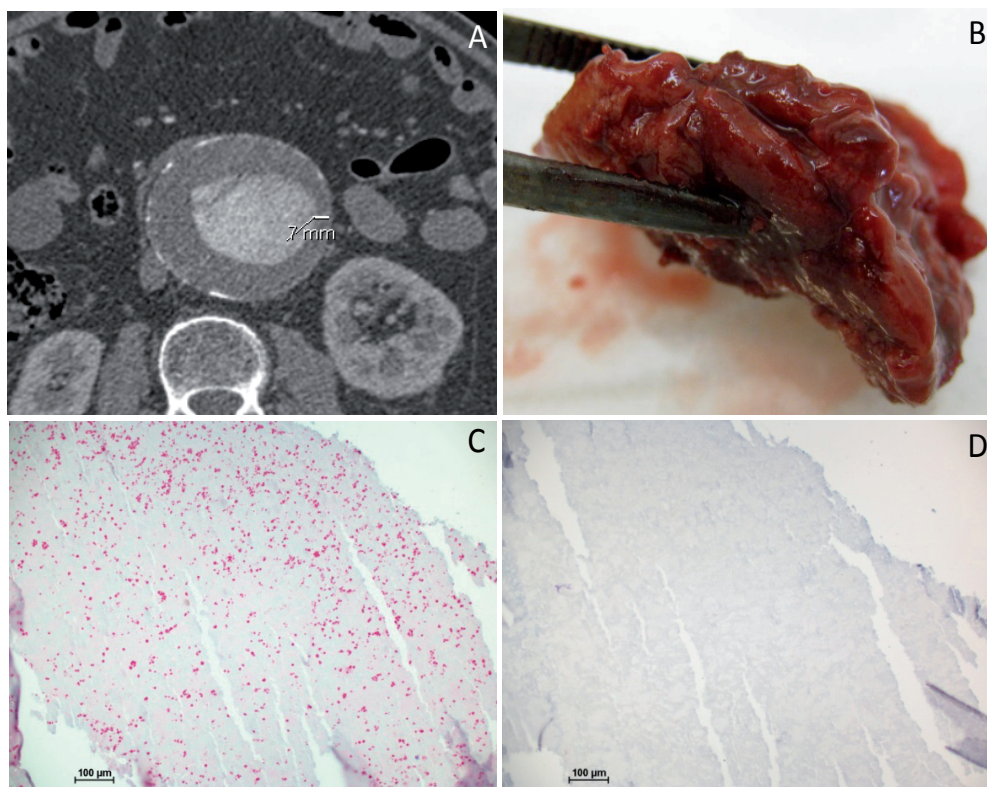


Figure 4: A thin ILT with many neutrophils. A) A CT image of this ILT before surgery. B) A photograph of the thin part (7mm) of the ILT after surgery C) the thin ILT stained with antibodies against CD66acd, a marker for neutrophils, and D) a negative control stain, in which we omitting the primary antibody.

In a thick ILT the luminal layer contains numerous CD66acd-positive neutrophil leucocytes, CD61 positive platelets and to a lesser extent macrophages (CD163) and mast cells (identified by tryptase).

Concentrations of NE and MMP9 and their inhibitors

We measured concentration of MMP1, MMP2, MMP9, MMP13 and neutrophil elastase (NE) in all three layers of the ILT. We also measured concentrations of inhibitors of some these proteases, TIMP1, and α -1 anti-trypsin and also PAI-1. The amounts of MMP1 and MMP13 were very low in all layers of the ILT. MMP2 was present with an intermediate amount while MMP9 and NE were present in the luminal layer, away from the AAA wall, with smaller amounts in the middle and abluminal layers (Table1).

Table 1

Concentrations and activity of enzymes and their inhibitors in luminal, middle and abluminal layers of the intra-luminal thrombus.

	Luminal	Middle	Abluminal
Proteases			
MMP-1	36 (25; 60)	36 (18; 62)	22 (20; 39)**
MMP-2	158 (99; 610)	98 (67; 249)	70 (42; 114)***
MMP-9	3459 (658; 6305)	492 (100; 1148)	278 (150; 1370)***
MMP-13	1.1 (0.3; 3.25)	0.39 (0.1; 1)	0.5 (0.3; 1.1) ^{ns}
NE	26577 (2507; 63740)	836 (274; 3496)	599 (155; 3050)****
Inhibitors			
TIMP-1	1628 (650; 4100)	1851 (904; 3027)	2762 (1663; 4731)**
PAI-1	45 (16; 108)	55 (26; 96)	79 (27; 139) ^{ns}
α 1-Antitrypsin	1170 (298; 1880)	350(0; 720)	520 (0; 830) [†]
Protease activity			
MMP-9 activity	5.4 (3.4; 9)	2.8 (0.6; 14)	0 (0; 0)**
NE activity	83 (5; 244)	1.96 (0.6; 38)	2.7 (0.4; 57)***

All values, median (interquartile range) are given in ng/g wet weight of the tissue. Asterisks denote levels of significance comparing results from luminal and abluminal layers. ^{ns}Non significant.

* $p \leq 0.05$.
 ** $p \leq 0.01$.
 *** $p \leq 0.001$.
 **** $p < 0.0001$.

As seen in Table 1, concentrations of proteases and their inhibitors that are present in the different layers of the ILT varies. MMP9, NE, TIMP1 and α -1 antitrypsin were present in all layers of the ILT with high concentrations. Although the mere concentrations of the proteins are inconclusive, but when different layers of the ILT were compared, we observed that the amount of these proteins in the luminal layer of the ILT is significantly higher than in the abluminal layer. Since the luminal layer is in direct interface with the blood, it could be questioned if these proteins enter the ILT from circulating blood? To answer this question we measured the concentration of all these proteases and inhibitors in plasma of the same patients cohort. The results showed that the amount of these proteins in plasma did not correlate significantly with the proteins measured in the luminal layer of the ILT.

Activity of NE and MMP9

The two proteases selected for activity measurements, MMP9 and NE, were assayed using specialized kits. We assayed samples from all layers of ILT and plasma of our cohort. The results showed that there is a measurable activity in the luminal layer of the ILT while it differed significantly from that in the abluminal layer, where the activity was very low or zero. The activity in the plasma was higher but again was not correlated with that in the luminal layer of the ILT, the layer in contact with the blood. The lack of activity in



the abluminal layer of the ILT could be explained by presence of inhibitors in this region, where their concentrations were significantly higher compared to the luminal layer.

NE and MMP9 are inhibited

Further evidence for inhibiting the activity of MMP9 was obtained by immunoblot. Samples of AAA tissue were homogenized and protein extracts of these homogenates were assayed for TIMP1 in an immunoblot in non-reducing condition. The blot showed TIMP1 both in complex with another molecule at 135 kDa and also free TIMP1 with a band below 34 kDa. TIMP1 is a protein of 25 kDa that binds to MMP9 in a 1:1 manner and inhibits its activity. When we calculated concentrations of these proteins using simple stoichiometry in the abluminal layer, we found several fold excess of TIMP1 compared to MMP9. Thus we concluded that activity of MMP9 is inhibited by TIMP1 in the abluminal layer of the ILT, this explained the lack of activity in our activity measurement assay, despite our previous finding of presence of N-GAL and the zymography assay that showed activity of MMP9 in a few samples. The discrepancy could be explained by the fact that in activity assay we sampled more biopsies than in the Zymogram. There may also be differences in the sensitivity of the two methods, whether the observed gelatinase activity identified by the zymography method is of functional relevance remains to be shown. An additional explanation is that our method in paper I of co-immunoprecipitation, treatment with SDS and a reducing agent, electrophoresis, and then zymography would have separated MMP9 complexes from TIMP1.

Proteolytic activity is limited to the surface of the ILT

Adolph et al. (Adolph, Vorp et al. 1997) studied the possibilities for cells from the blood stream to penetrate the ILT and be transferred to the vessel. They concluded that trans-thrombotic cell migration occurs but is limited to 10 mm depth from the outer surface of the luminal layer of the ILT. This finding explains why the abluminal layer of the ILT is almost devoid of live cells. This is why we found a significant difference in the concentration and activity of proteases between luminal and abluminal layer of the ILTs that are thicker than one centimeter. In the abluminal layer we found a negative correlation between activity of NE and the volume of the ILT. This indicates that the thicker the ILT, the less activity of NE is measured in the abluminal layer of the ILT. Further evidence comes from a study that showed the AAA wall under a thin ILT contains more active MMP9 and NE compared to the wall covered by a thick ILT (Wiernicki, Stachowska et al. 2010).

Take-home message

Summary of data generated in this study indicates that the luminal layer of the ILT contains several types of soluble proteases and cells, such as neutrophils, that are capable of producing proteases. The proteolytic activity of these soluble proteases, however, seems to be largely blocked by the presence of endogenous inhibitors. This is in agreement with the work of Carrel et al. where it was shown that most MMP9 in the abluminal ILT is latent (Carrel, Burnand et al. 2006). The findings in papers I and II led us to examine an entirely different class of candidate proteases in paper III.

Paper III

(Microvesicles as carriers of membrane-bound proteases)

Thus far, we had not found a plausible explanation for the aortic wall under the ILT (TVW) being more degraded than the wall “free from ILT”(NTVW). In our previous studies, we found very little proteolytic activity of N-GAL/MMP9, total MMP9, or neutrophil elastase in the abluminal layer of the ILT, adjacent to the TVW. In paper III, we focused on a different class of proteases, transmembrane proteases that might take part in the pathogenesis of AAA.

We were particularly interested in the possibility that these transmembrane MMPs could reside on membrane microvesicles (MVs), which are bilayer structures that are released from cells upon activation or apoptosis. MVs transport biologically active molecules, and often survive longer and diffuse more widely than their parental cells (Giesen, Rauch et al. 1999; Liu and Williams 2012). Thus, we hypothesized that they might persist within the abluminal layer of human ILT, which is virtually devoid of cells.

Membrane bound proteases (background)

Two major members of the transmembrane class of proteases are ADAM10 (a disintegrin and metalloprotease-10) and ADAM17. ADAMs have overlapping substrate specificity as soluble metalloproteases and have been shown to function as a gelatinase (Millichip, Dallas et al. 1998) and are able to target several matrix proteins, such as cadherins and collagen type XVII (Edwards, Handsley et al. 2008). ADAM17 possesses TNF α converting enzyme (TACE) activity, meaning that it cleaves pro-TNF α into the active cytokine. TNF α has been shown to be present in the AAA aorta and expressed by macrophages, in contrast to control aorta (Sato, Nakamura et al. 2004). Other substrates of ADAM17 include syndecan-1 and -4, ICAM-1, VCAM-1, and collagen type XVII, which is a vascular matrix protein that may participate in pathogenesis of AAA (Scheller, Chalaris et al. 2011; Zheng, Tian et al. 2012). Both ADAM10 and 17 are sheddases, which means that they are capable of cleaving off the ectodomain of membrane proteins for release. Studies in vitro show that some ADAMs are able to shed the ectodomain of other ADAMs, to release a soluble, enzymatically active fragment (Parkin and Harris 2009). ADAM17 was found to be significantly up-regulated in the media and adventitia of the aortic wall of AAA patients compared to controls (Sato, Nakamura et al. 2004), while tissue inhibitors of metalloproteinases (TIMPs) that inhibit ADAMs were shown to be significantly down-regulated in those regions (Lipp, Lohoefer et al. 2012).

ADAM10 and ADAM17 mRNA are present in the adventitia and media of AAA

In this study, aneurysmal vessels obtained at elective, open AAA repair were separated into media and adventitia, total RNA was extracted from these layers, and cDNA was produced. Real time qPCR with primers recognizing ADAM10 and ADAM17 were used to measure the amount of these mRNAs in the samples. The results showed that most of the ADAM10 and ADAM17 mRNAs were present in the media of the aortic wall of AAA, with some expression in AAA adventitia.



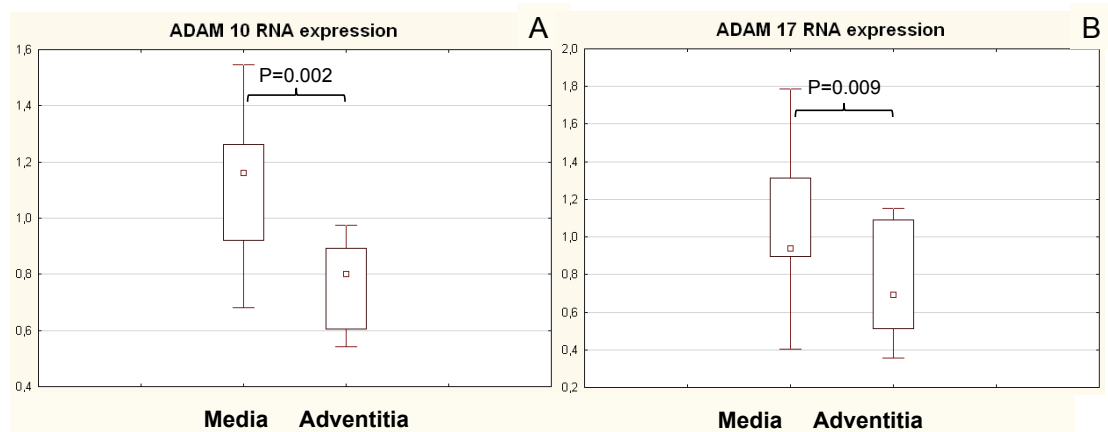


Figure 5: Levels of ADAM10 (panel A) and ADAM17 (panel B) mRNAs in media and adventitia of AAA aortas, as assessed by real-time qPCR. Displayed are medians (squares), middle two quartiles (boxes), and ranges (whiskers), $n = 5$ NTVW and 11 TVW. Values were normalized to RPLP0, P-values were computed using the Mann-Whitney rank-sum test.

This shows that aortic tissue does have cells that express ADAM10 and ADAM17 and that the amount of mRNA expressed in the media is significantly higher than that in adventitia. The predominant cells in the media are smooth muscle cells. However there are some other cells types also seen in the aortic tissue of the aneurysm such as neutrophils and macrophages.

ADAM10 and ADAM17 protein are present in the cell-free abluminal layer of the ILT, adjacent to the AAA wall

We stained human ILT and AAA samples with antibodies that recognize the extracellular domains of ADAM10 and ADAM17. We found that both proteases were present in these samples. Interestingly, both ADAM10 and ADAM17 were readily seen in the cell-free abluminal layer of the ILT samples. The abluminal layer is the layer directly adjacent to the vessel wall, and any proteases residing in this layer could potentially damage the underlying AAA wall.

Since ADAMs are membrane-bound proteases, and we found that they are present in an environment where cells are absent, we hypothesized that these transmembrane proteins may exist on membrane microvesicles. Microvesicles (MVs) are membrane blebs that bud off from activated or apoptotic cells. All cells in the body under certain conditions will produce MVs. Radiation, chemical insults including cigarette smoke, and lipopolysaccharide can provoke the release of MVs by cells. By budding off from the cell surface, MVs can carry proteins that were embedded in the plasma membrane of the cells of origin. In this way, membrane-bound proteins can exist in a cell-free environment. Importantly, MVs can often diffuse farther and persist longer than their cells of origin (Liu and Williams 2012).

Microvesicles in ILT and AAA wall seen by transmission electron microscopy (TEM)

We performed electron microscopy to determine if the characteristic acellular bilayer structure of MVs was present in ILT or the AAA wall. Biopsies from ILT and AAA aorta were fixed in paraformaldehyde and prepared further for TEM (see material and method). The luminal layer of the ILT, which faces the blood flow, contained cells embedded in a mesh of fibrin and red blood cells. In this layer, our TEM images did not show MVs. In contrast, the abluminal layer of the ILT and the AAA wall showed abundant numbers of acellular membrane structures $< 1 \mu\text{m}$ in diameter, consistent with MVs (Figure 5).

To determine if these membrane MV structures carried transmembrane proteases, we used immunogold staining, i.e., staining with gold particles of two different sizes linked to antibodies against ADAM10 and ADAM17. These TEM images showed MVs with membrane-bound ADAM10 and ADAM17. By co-staining with antibodies linked to two different sizes of gold particles, we found by TEM that a subset of these ADAM10- and ADAM17-positive microvesicles appeared to originate from neutrophils (CD66acd-positive) and another subset from platelets (CD61-positive) (Figure 6).

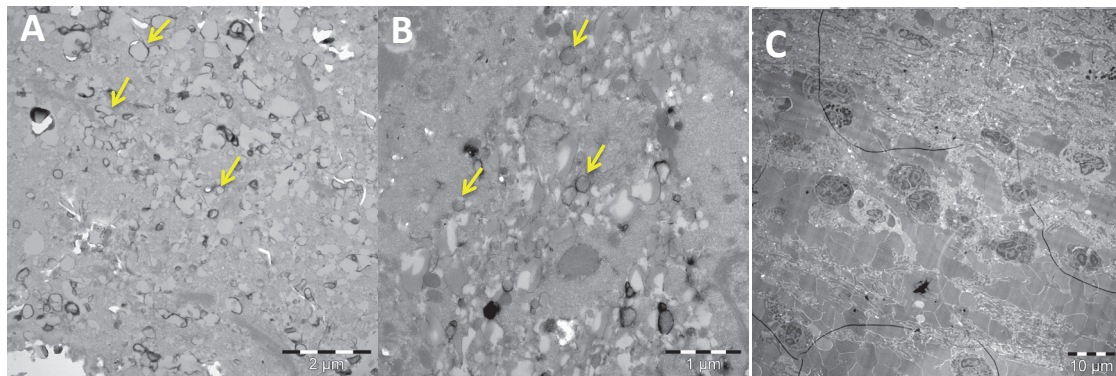


Figure 5: The presence of MVs in AAA tissue.

A shows aortic wall of AAA, B is abluminal layer of ILT and C is luminal layer of the ILT where MVs are absent. Yellow arrows in A and B show MVs.



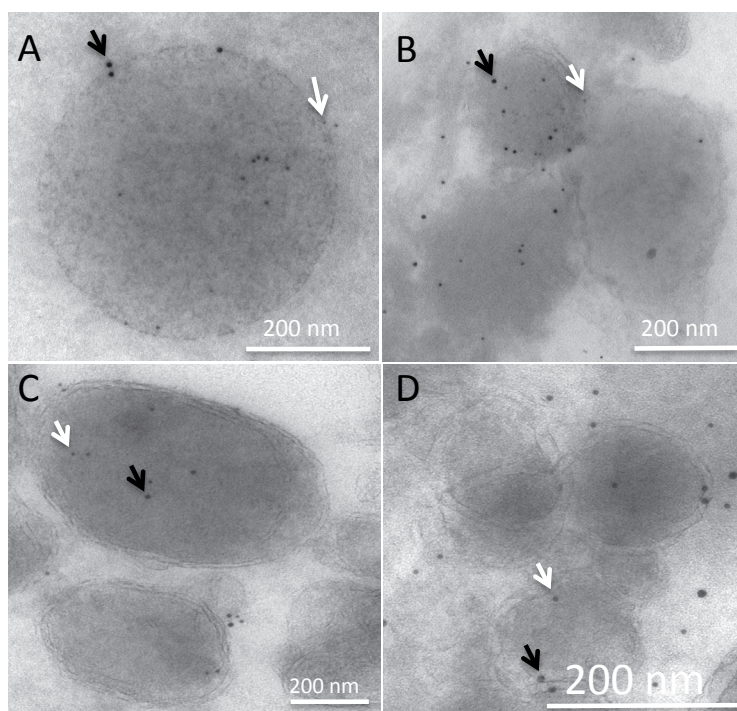


Figure 6: Double goldstaining transmission electron microscopy. Immunogoldstaining with ADAM10 (black arrow, bigger spots) and platelets marker, (white arrow, smaller spots) A+C. ADAM17 (white arrow, smaller spots) and neutrophil marker (bigger spots, black arrow) B+D. A and B in abluminal ILT and C and D in aorta.

Cellular origin of MVs with ADAMs on their membrane

We investigated the cellular origin of ADAM10 and ADAM17-positive MVs by yet another method. We used flow cytometry of fixed extracts of the abluminal layer of human ILT and aorta. To detect MVs, we gated for events sized from 200nm to 1 μ m that were also positive for annexin V. Annexin V is a molecule that binds to phosphatidylserine exposed on the exofacial leaflet of membrane bilayers (Montoro-Garcia, Shantsila et al. 2012). In normal cells, phosphatidylserine is asymmetrically concentrated in the inner leaflet of the plasma membrane bilayer. This asymmetry is disrupted in MVs. Activated platelets and apoptotic cells, however, can also display phosphatidylserine on the exofacial leaflet that can bind to annexin V; hence, a major use for annexin V is the detection of apoptotic cells.

Our size gating was chosen to distinguish MVs, which are small, from activated platelets and apoptotic cells (Montoro-Garcia, Shantsila et al. 2012). The two antibodies we used to stain the flow cytometry samples were anti-ADAM10 and anti-CD66 (a marker of neutrophils) in one subset, and anti-ADAM17 and anti-CD66 in another subset. The results from these flow cytometry experiments (Paper III, figure 4) show abundant neutrophil-derived MVs that also carry ADAM10 and ADAM17. There were consistently more neutrophil-derived MVs carrying ADAM10 than carrying ADAM17.

We have also initiated flow cytometry to detect platelet-derived MVs that carry ADAM10 or ADAM17.

Enzymatically active ADAM17 in AAA tissue

Because we found ADAM10 and ADAM17 protein in the abluminal layer of the ILT and in aortic wall of AAA, we investigated the enzymatic activity of these proteases. The TACE activity of ADAM17 was easily detected in all layers of the ILT and also in the AAA aorta (Figure 7). Activity measurement of ADAM10 however requires assay development due to lack of certified commercially available assays and we are currently working on it.

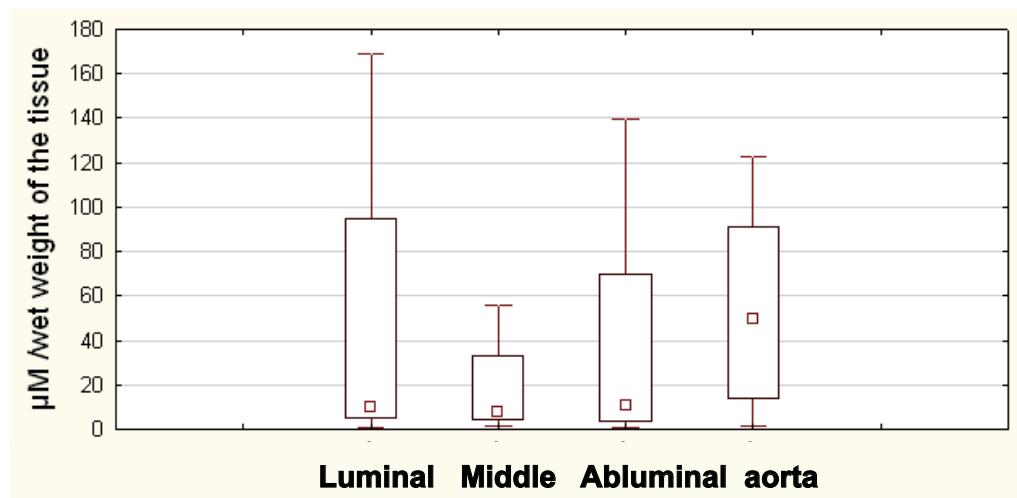


Figure 7: Enzymatic activity of ADAM17 measured in layers of the ILT and in AAA wall

Neutrophils exposed to tobacco smoke extract (TSE) release MV carrying active ADAMs

Because tobacco smoking is the leading risk factor for the development of human AAA, we hypothesized that transmembrane proteases and MVs in our ILT samples could have arisen as a consequence of smoke exposure. To test this hypothesis in vitro, we established a cell-culture model by exposure of human HL-60 neutrophils to TSE. We found that TSE exposure induced ADAM10 and ADAM17 expression by human neutrophils in a dose- and time-dependent manner, shown by immunoblots of cellular extracts. Moreover, TSE stimulated the cleavage of these proteases into their active forms. Exposure to TSE induced apoptosis of these cells, detected by TUNEL staining, in a time-dependent manner. Most importantly, MVs isolated from supernatants of cultured human neutrophils after 24h of TSE exposure carry active ADAM10, detected by immunoblots of the MV fraction from conditioned media (Figure 5, paper III).

Take-home message

The studies in paper III demonstrate the presence of significant amounts of enzymatically active transmembrane proteases in the abluminal layer of the ILT and within the AAA



aortic wall. Thus, unlike the soluble proteases that we studied in papers I and II, these transmembrane proteases are plausible participants in the pathogenesis of AAA, because they are abundant, active, and located in or directly adjacent to the damaged aortic wall. These transmembrane ADAM proteases are present on MVs, which are membrane blebs that can persist long after their parental cells have disappeared. The presence of MVs in AAA tissue had been previously shown, but only in relation to transport of tissue factor and renewal of the ILT (Touat, Ollivier et al. 2006).

Here, we showed that MVs can carry proteases and might appear as a result of cell death in AAA tissue. Tobacco smoke, the prime environmental risk factor for AAA, stimulated ADAM10 and ADAM17 expression by a human neutrophil cell line in culture, apoptosis of these cells, and the generation of ADAM-positive MVs that resemble the MVs that we found in the ILT and AAA wall. The results present a compelling pathophysiologic model, in which tobacco smoke stimulates immune cells to produce ADAM10 and ADAM17 and then export these enzymes on long-lived MVs that persist in the abluminal layer of the ILT and the AAA wall. Active soluble MMPs degrade the wall, and ADAMs may take part in degrading matrix component of the wall of AAA and also activate TNF α (via TACE activity), a cytokine that recruits additional immune cells that further damage the aneurysm.

Ongoing project (elastase does many things)

Diameter of AAA together with its growth rate is the only measure available today to evaluate the risk of rupture (Vardulaki, Prevost et al. 1998). To date there is no plasma tests that can predict the risk of rupture. Most AAAs produce ILT, which predominantly is made of fibrin that is a result of coagulation activity. The fibrin produced is degraded later during a process called fibrinolysis that degrades fibrin in healthy situations. Plasmin is the predominant protease in this process. However in AAA patients this process is halted to certain degree due to unknown reason and ILT grows in volume continually but also degrades to certain extent. Degraded ILT predisposes the AAA to rupture. This has been shown in plasma by measuring the elevated level of D-dimer of AAA patients and its significant correlation to growth rate of AAA.

Neutrophil Elastase causes Fibrinolysis of the Intraluminal Thrombus of Abdominal Aortic Aneurysm

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Introduction

AAA patients experience few or no symptoms during disease progression. Aneurysm diameter can be measured by ultrasonography (US), which is a non-invasive procedure. US can provide information about risk of rupture by determining if the aneurysm diameter is equal to or exceeds 5.5 cm (2002). AAAs often have an intraluminal thrombus (ILT)

Hans, 2005 #188}, the growth of which is associated with AAA diameter (Parr, McCann et al. 2011), implying that most AAAs with a diameter indicating risk of rupture contain an ILT. The ILT has been reported to be of importance for the pathogenesis of AAA (Roy, Labruto et al. 2006).

Neutrophil leucocytes and platelets are found in the luminal layer of the ILT (Houard, Touat et al. 2009). Neutrophils contain elastase (NE), a serine protease released from the azurophilic granules that is capable of degrading elastin. In addition, NE has fibrinolytic activity resulting in fibrin degradation products (FDPs) with a specific epitope (Kohno, Inuzuka et al. 2000). Cathepsin G also released from neutrophil leucocytes has fibrinolytic activity, but with much lower potency compared to NE (Kolev, Komorowicz et al. 1996). NE also promotes fibrinolysis indirectly by cleaving plasminogen resulting in mini-plasminogen (Wohner 2008).

AAA rupture occurs most often in the aneurysm wall covered by a mural thrombus (Simao da Silva, Rodrigues et al. 2000). In support of this, bleeding into the ILT identified as a crescent sign visible on axial images with contrast enhanced CT indicates risk of rupture (Arita, Matsunaga et al. 1997). Bleeding into the ILT is significantly more common in patients with ruptured compared to intact AAAs (Roy, Labruto et al. 2008). The entrance of blood into the ILT is most likely facilitated by fibrinolysis, which permits bleeding to reach the underlying aneurysm wall.

The present study evaluates NE and plasminogen derived FDPs in aneurysm patients scheduled for elective open or endovascular repair surgery. The purpose of this study is to evaluate the risk of rupture by measuring levels of NE derived FDPs in plasma of patients with AAA.

Patients and Methods

Seventy patients, 59 men and 11 women, undergoing elective surgery for AAA were included in the study, the mean age was 70.4 years, 70 years for men and 71 for women. Blood samples anti-coagulated with citrate obtained prior to surgery were centrifuged 20 min. at 2500×g followed by 30 min. at 20,000×g to obtain platelet free plasma.

ILT volume was measured on CT images in 59 cases using the volume program of Siemens Leonardo Workstation. The selected Hounsfield unit settings were dynamic in order to exclude the contrast filled lumen, but still include the complete ILT. Missing measurements were due to unavailable CT images performed at other hospitals, no contrast, or too thick CT-slices.

The study was approved by the regional ethics committee and participants gave their informed consent.

Plasma levels of elastase-digests of cross-linked fibrin (elastase XDP) were determined with a sandwich ELISA, essentially as described by Kohno et al (Kohno, Inuzuka et al. 2000). As in the original publication we used the monoclonal antibody anti-elastase XDP clone IF-123 from Cosmo Bio Co. Ltd (Tokyo, Japan) as capture antibody, and horseradish peroxidase-labeled anti-human fibrinogen rabbit antibody from DAKO (Glostrup, Denmark) as probing antibody. The inter-assay coefficient of variation of 7% (n=10) was calculated using samples of coagulation reference plasma from Technoclone



GmbH (Vienna, Austria).

The capture antibody IF-123 has been shown to specifically bind granulocyte elastase digest products of fibrinogen/cross-linked fibrin, and not to bind plasmin digests of either substrate. For further characterization of the antibody IF-123, we incubated fibrinogen (3 μ mol/L) with elastase (Product E-8140, Sigma), plasmin or reaction buffer alone (0.05M Tris, 0.15M NaCl, pH 8.0), proteases at 88nmol/L. Samples were taken with minutes intervals for 1 hour, and treated with excess of protease inhibitor cocktail (complete, mini, EDTA-free, from Roche). Samples were thereafter analyzed for reactivity in the ELISA described above. Incubation of fibrinogen with elastase generated product(s) that reacted in the ELISA with IF-123 (seen as increase in OD in the samples taken after 40 minutes incubation), which did not occur with incubation with plasmin or buffer alone, (Figure 8).

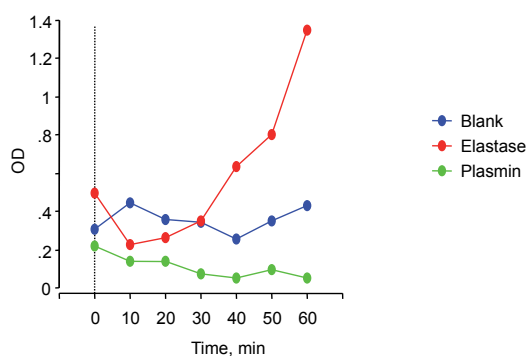


Figure 8: Evaluation of XDP recognizing antibody.

Results

A positive correlation between ILT volume and elastase XDP $r = 0.58$, $p < 0.05$ and D-dimer $r = 0.53$, $p < 0.05$ was found. No similar significant correlations were seen for AAA diameter. There was a strong positive correlation between plasma D-dimer and elastase XDP $r = 0.85$, $p < 0.05$. After surgery there was a significant decrease of plasma elastase XDP compared to plasma levels of XDP before surgical AAA repair (Figure 9). Volume of the ILT is associated with aneurysm diameter, but there were significant correlations although weak, for elastase XDP and D-dimer with aneurysm diameter in the present study $r = 0.18$ and 0.16 respectively.

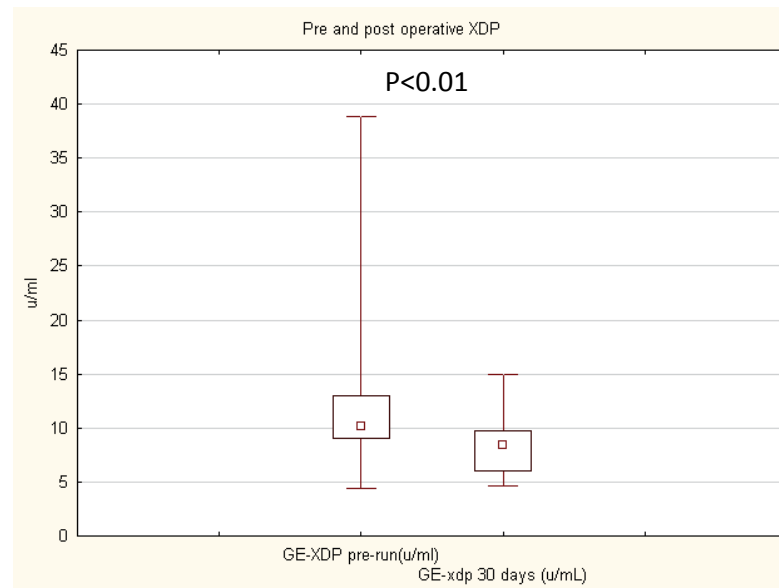


Figure 9: Pre and postoperative levels of XDP: Box-and-whisker plot showing plasma levels of elastase derived fibrinolysis products (XDP) before and after elective open repair surgery for AAA. P-values were computed using the Mann-Whitney rank-sum test, (n=11)

Discussion

The present study shows increased elastase XDP levels in plasma, reflecting neutrophil elastase (NE) activity in the ILT. Neutrophils are readily found in the canaliculi of the ILT (Figure 10), and are a source of proteases to further degrade the ILT and the aneurysm wall.

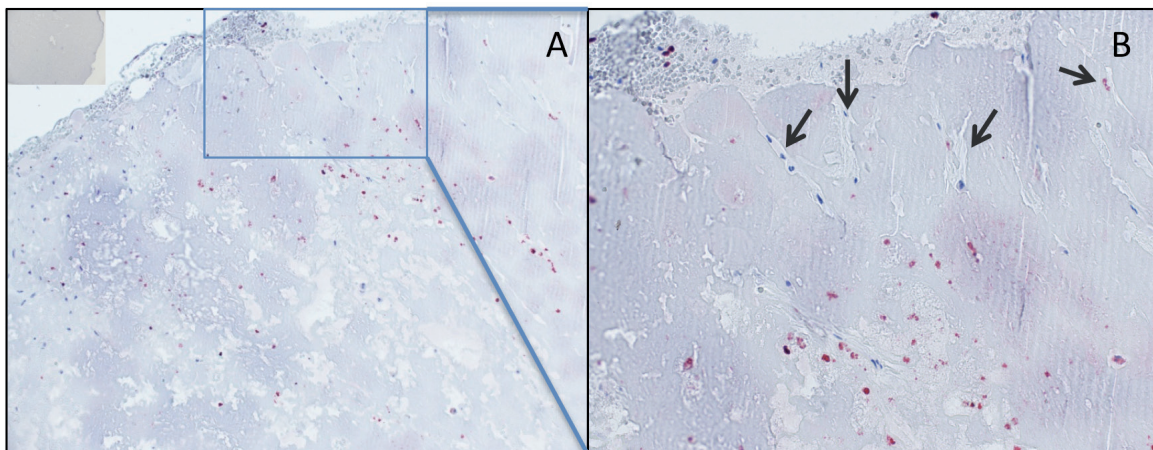


Figure 10: Luminal layer of the ILT stained with neutrophil marker CD66acd (red) and haematoxylin counterstain (blue). A) Image shows 10 x magnification with negative-control image in the corner (no primary antibody). B) Image shows 20 x magnification where arrows show canaliculated tissue that allows entrance of the cells into the ILT.



NE also promotes plasmin activity by converting plasminogen into mini- plasminogen, which is more readily activated by plasminogen activators. Furthermore NE inhibits α 2-antiplasmin and PAI-1 (Lerant, Kovacs et al. 1990). Consequently the correlation between elastase XDP and D-dimer can be increased by plasmin fibrinolysis induced by NE. Accordingly it could be suspected that NE contributes to an increased level of D-dimer, which has been reported to be a marker for presence and growth rate of AAA (Golledge, Muller et al. 2011).

The ILT is not strong enough to withstand pulsatile stress (Gasser, Gorgulu et al. 2008). Bleeding into the ILT allowing blood flow to reach the underlying aneurysm wall is likely based on weakness of the ILT caused by fibrinolysis. In addition to NE, degradation of fibrin is caused by other neutrophil derived enzymes namely cathepsin G (Kolev, Komorowicz et al. 1996) and membrane type 1-MMP (Kluft 2003), the latter originates from neutrophil microvesicles, which have been found in the ILT (Own unpublished observations).

The AAA wall covered by a thick ILT is thinner and less resistant to wall stress compared to wall segments with only a thin or not macroscopically detectable ILT (Vorp, Lee et al. 2001). Identification of bleeding in the ILT by localized areas with higher attenuation in coronal, axial and sagittal projections have been shown in significantly more cases with ruptured compared to intact AAAs (Roy, Labruto et al. 2008). Accordingly fibrinolysis in the ILT amplified by NE could increase the risk of AAA rupture.



CONCLUSION

In this thesis, we concentrated on the ILT of AAA. Previous studies have suggested that the ILT could play an active role in AAA development. Because the ILT is present in most large AAAs, its effect on the underlying vessel wall should be considered. The biomechanical effects of the ILT have been shown to be important in calculating the risk of AAA rupture. The presence of proteases in the ILT and the aortic wall was the main concern of this thesis. We started with MMP9 and its binding partner, N-GAL that protects it from degradation. In paper I, we could show that they form a complex in AAA tissue, which could lead to preserved activity of MMP9.

In paper II, we investigated the presence of soluble proteases and their inhibitors in the ILT, to try to explain the reason for the aortic wall under the ILT (TVW) being thinner and more degraded than the wall that is not under the ILT (NTVW). Proteases, such as neutrophil elastase and MMP9, have been shown to play active roles in the pathogenesis of AAA. Nevertheless, we found that the activity of these proteases in the abluminal layer of the ILT was very low or close to zero. This could be the result of inhibitors such as α -1 anti-trypsin and TIMP1. These inhibitors are present in the ILT with higher concentrations in the abluminal layer of the ILT compared to the other layers. This was the layer where the activities of soluble proteases were shown to be the lowest.

In this process we have learnt that there might not be such thing as ILT free wall in our samples but aortic wall exposed to ILT in different length of time. The noteworthy point that should be kept in mind here is the fact that the sample collection was only available at the later stage of the disease.

Observing these aorta segments in a microscope, (Figure 1), we noticed that there always exist some ILT covering the aortic wall, even the one considered free from the ILT deduced by CT-images. This wall is the wall shown to have a higher mRNA expression of MMPs than the wall covered with the ILT in previous study by our group. The proteases in the luminal layer of the ILT are the most active. This layer resembles the thin thrombus that covers the so called “thrombus free wall”. Investigating this wall may be important for the understanding of what happens during the early stages of AAA development.

In paper III, we examined a physically distinct class of proteases, the transmembrane ADAM proteases. We found that ADAM10 and ADAM17 (also known as TACE) are abundant and enzymatically active in the ILT and AAA aortic wall. Importantly, ADAMs are present in the abluminal layer of the ILT, immediately adjacent to the aortic wall. We found that these molecules are embedded in the membrane of MVs that had bud off from cells in AAA tissue. Neutrophils and platelets are among the cells that produce proteolytically active MVs in AAA tissue. Interestingly, exposure of neutrophils in vitro to tobacco smoke extract induced the production of MVs that carry active ADAMs on their membrane.

In an ongoing project investigating the effect of NE on fibrin mesh of ILT, we found a significant correlation between elastase-digests of cross-linked fibrin (elastase XDP) in plasma of AAA patient and volume of the ILT. Further investigations are required to determine levels of XDP in plasma of AAA patients compared to the control plasma.

Overall, we have shown in this thesis that the ILT may have a role in the pathogenesis of AAA. The ILT stores cells and proteases in its mesh and could thereby damage the underlying aortic wall.

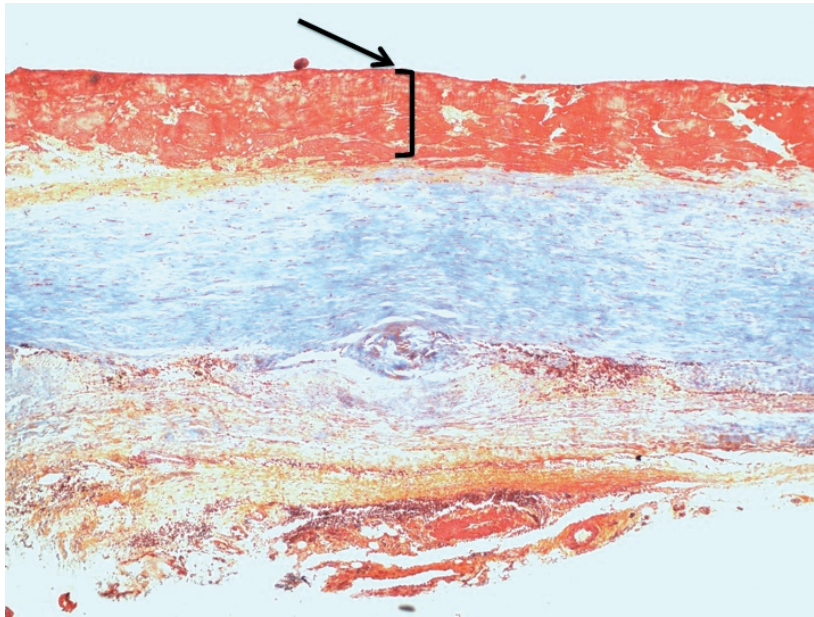


Figure 1: Aneurysmal aorta free from the ILT stained with Masson's Trichrome where muscles that stains red are not present but over production of collagen in light blue is seen. The top layer that is red and is indicated by the arrow shows a thin layer of ILT covering what is left of the endothelial layer, 10x magnification.



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